Nucleic acid and protein metabolism in yeasts, higher plants and plant virus infections

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Thesis presented for the degree of Doctor of Science

University of Edinburgh

1982

ABSTRACT

Research on nucleic acid and protein metabolism in yeasts, higher plants and in viral infections of higher plants is described.

The antibiotic lomofungin, and 8-hydroxyquinoline were studied as inhibitors of RNA synthesis in yeast, and their mode of action was shown to be by chelation of bivalent cations required for RNA polymerase activity. The turnover of messenger RNA in yeast was studied using 8-hydroxyquinoline, and a mechanism of translational control over yeast protein synthesis proposed.

Research on rates of ribosomal and messenger RNA synthesis in fission yeast (Schizosaccharomyces pombe) and budding yeast (Saccharomyces cerevisiae) suggested that for both species, synthetic rates of both types of macromolecule doubled after the period of DNA synthesis in each cell cycle. A simple model relating DNA content (gene dosage), rate of transcription and observed patterns of enzyme accumulation during the cell cycle was proposed for S. pombe. The model was tested and elaborated by study of mutant cells differing in their genetic control over cell size; by examining haploid and diploid cells, and by a mathematical simulation. The results suggested that a cell-size related control operated over the rate of transcription, and could be important in control of cell growth rate and balanced exponential growth.

Experiments with the higher plant cell cycle used the synchronous cell divisions induced in explants of Jerusalem artichoke tuber by excision and culture. Freshly cut explants contained polyadenylated messenger RNA and ribosomal RNA. Messenger RNA synthesis predominated

in the first few hours of culture; later, ribosomal RNA was accumulated in a stepwise manner, but the periodicity was shown not to be cell-cycle related. Ribosomal RNA synthesis was not required for induction of cell division or for the net accumulation of protein that occurred during the first two divisions. Two periods of putative messenger RNA synthesis were identified which were required for the occurrence of the succeeding divisions.

Tobacco mosaic virus RNA was shown not to be polyadenylated, and not to have detectable methylation. A previous report of nucleoside residues with cytokinin activity in this RNA could not be confirmed.

Tobacco mosaic virus infection of tobacco plants inhibited synthesis and decreased stability of chloroplast ribosomal RNAs. Synthesis of cytoplasmic ribosomal RNA was initially stimulated, but was inhibited during active virus multiplication. Cytoplasmic ribosomal RNA stability was increased by infection. Virus infection reduced the rate of host protein synthesis by 75%, but did not alter the host polyadenylated messenger RNA content, suggesting a control at the translational level. Changes in concentration of abscisic acid were measured after infection, and shown to be important in control of growth and RNA metabolism in infected plants.

Constitutive resistance to tobacco mosaic virus controlled by the Tm-1 gene in tomato, and induced resistance to the virus in tobacco were studied, and information gained on the mechanism involved in each resistance. Methyl benzimidazol-2yl carbamate was found to inhibit tobacco mosaic virus multiplication in tobacco.

RNA metabolism was studied in germinating S. pombe ascospores, and in germinating tobacco and carrot seeds.

Methods were developed for detection of polyadenylated messenger

RNAs, and for radioassay of abscisic acid in plant extracts.

A review article discussed the involvement of plant growth regulators in control of plant-virus interactions.

CONTENTS

1. Publications on nucleic acid metabolism and the cell cycle in yeast

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ACKNOWLEDGEMENTS

In research, support comes from many quarters. I am grateful to technical assistants for careful and reliable work, to colleagues for their involvement in collaborative research and stimulating arguement, and to directors of institutes and heads of departments for facilities, advice and encouragement. In particular I wish to thank Professors R. Brown FRS and M. M. Yeoman, and Dr U. E. Loening of the Botany Department, University of Edinburgh; Professors G. Melchers, K.-W. Mundry and S. Sarkar, and Frl. Ute Klöpfer, of the Max-Planck-Institut für Biologie, Tübingen; Professor J. M. Mitchison, FRS and Drs P. Nurse, J. Creanor, F. Moreno and B. L. A. Carter of the Department of Zoology, University of Edinburgh; and Professor J. K. A. Bleasdale, Mrs S. A. R. Loughlin and Messrs R. Whenham, A. Gerwitz and A. Barnes of the National Vegetable Research Station.

Financial support for the work described in this thesis was provided by the Sir David Baxter Scholarship Fund of the University of Edinburgh; the Max-Planck-Gesellschaft zür Förderung der Wissenschaft; the Medical Research Council and the Agricultural Research Council, and is gratefully acknowledged.

INTRODUCTION

During a research career, the areas of science one studies can change considerably. Partly this is due to evolution and broadening of interests, and to opportunities opened by new contacts or techniques. Partly it can be a result of the demands of geography and employment. The papers in this thesis deal with a wide range of living organisms: bacteria, yeast, higher plants and a mammal, as well as with plant viruses. The common denominator to the great majority of the papers is that they attempt to interpret biological processes, such as the cell cycle, or virus infections of plants, in terms of the molecular behaviour of nucleic acids and proteins. This introduction is intended to place the main subject areas in perspective.

The cell cycle in yeast

An understanding of the controls involved in the cell cycle is central to understanding growth and development of living organisms. In balanced exponential growth, such as is seen in logarithmic phase cultures of unicellular micro-organisms, there is the problem of the control of balanced duplication of all the tens of thousands of cell components in each cycle. With division at the end of the cycle, the two daughter cells produced are each identical to the mother at the same stage in the previous cycle.

The fission yeast Schizosaccharomyces pombe provides an ideal opportunity to study this problem. It does show balanced exponential growth in culture, and synchronously-dividing cultures are easily prepared (Mitchison and Vincent, 1965). It is normally haploid, which facilitates genetic manipulation, but diploid strains may also be

prepared (Nurse, Thuriaux and Nasmyth, 1976). It must be ranked as one of the simplest eukaryotes; the genome size is only four times that of the bacterium Escherichia coli (Mitchison, 1970).

The first part of the work on the yeast cell cycle included in this thesis was descriptive: measurement of the rates of synthesis of polyadenylated messenger RNA (poly(A)mRNA) and ribosomal RNA (rRNA) during the cycle (Fraser and Moreno, 1976). This paper also attempted to relate the observed changes in synthetic rate to DNA replication and published patterns of enzyme accumulation (Mitchison and Creanor, 1969). A parallel study dealt with synthetic rates in the budding yeast Saccharomyces cerevisiae (Fraser and Carter, 1976) in which the cell cycle is somewhat more complex than in S. pombe. Both organisms showed similar cell cycle patterns of macromolecular synthesis.

The early experiments on the S. pombe cell cycle produced a simple model for control of growth during the cell cycle, relating DNA replication (gene dosage), rate of mRNA synthesis, and rate of protein accumulation (Fraser and Moreno, 1976). However, it soon became apparent that mutants of S. pombe with alterations in the genetic control of cell size (Nurse, 1975) posed a serious challenge to this simple model. In particular, the half-normal size mutant wee 1-50 had a growth rate, measured as rate of protein accumulation per unit DNA, much lower than that of wild type. Clearly there was no rigid relationship between DNA content, rate of transcription and rate of protein accumulation.

Experiments on the cell cycle of wee 1-50, and on a series of diploid strains of different size, suggested that after DNA

replication, a higher rate of transcription is not activated until the cell reaches a critical size (Fraser and Nurse, 1978; Fraser and Nurse, 1979). This size, measured as mass per haploid genome, is the same for mutants and wild types of all sizes. A mathematical analysis (Barnes, Nurse and Fraser, 1979) suggested that such a control could be very powerful in regulation of the accumulation of cell components to give balanced exponential growth. Its attraction is perhaps increased by evidence that the cell cycle timing of DNA synthesis is also controlled by a separate cell size control (Nurse, 1975; Nasmyth, Nurse and Fraser, 1979).

It must be stressed that the proposed cell size control over transcription is not envisaged as the only level of control. It is probable that transcription of some of the genome could be under separate types of control. The size-related control may also be modulated or even over-ridden in response to environmental changes, or conditions forcing the cells away from balanced growth.

A pre-requisite for the analysis of the role of messenger RNA metabolism in control of the yeast cell cycle was an estimate of mRNA half life (Fraser, 1975). One approach to measurement of this was to use inhibitors of mRNA synthesis, but at the time the work was started no suitable inhibitors were available for yeast. Experiments with a new antibiotic, lomofungin (Fraser, Creanor and Mitchison, 1973) gave an understanding of its mode of action: chelation of bivalent cations required for RNA polymerase activity (Fraser and Creanor, 1975). This knowledge also led to selection of a more suitable inhibitor of RNA synthesis, 8-hydroxyquinoline (Fraser and Creanor, 1974).

Studies of messenger turnover and protein synthesis using these inhibitors led to the discovery of an apparent mechanism of translational control of protein synthesis in S. pombe, involving long-lived mRNA (Creanor, May and Mitchison, 1975; Fraser, 1975).

The cell cycle in higher plants

Studies of the cell cycle in higher plants are necessarily made more difficult by the greater complexity of the organism and by the greater technical difficulty in manipulating the system. A synchronously-dividing culture of cells undergoing true balanced exponential growth is very difficult to achieve. A significant step forward was the development of a system for culture of explants of Jerusalem artichoke tuber tissue (Yeoman, Dyer and Robertson, 1965). In this system the first few divisions are inherently synchronous (Yeoman and Evans, 1967).

An early part of the work in this thesis was a study of the effects of light on cell division (Fraser, Loening and Yeoman, 1967). As a useful by-product, this work permitted the percentage of cells undergoing the synchronous divisions to be considerably increased. Further work used the convenience of the aseptic tissue culture system for early experiments on the synthesis of rRNA and its precursors in higher plant tissues (Rogers, Loening and Fraser, 1970).

Analysis of rRNA and poly(A)mRNA metabolism during the induction and progress of synchronous division was initially descriptive, defining patterns of accumulation and synthesis (Fraser and Loening, 1974; Fraser, 1975b). Further experiments with inhibitors of various types examined whether the observed changes were cell cycle-related events (Fraser, 1975a; 1975b. Fraser and Loening, 1974). Generally it was concluded that the accumulation of RNA and protein observed

during the early stages of culture did not depend on the occurrence of DNA synthesis and cell division, and that the periodic pattern of RNA accumulation was probably not cell cycle-related. Further experiments with inhibitors probed the extent to which cell division and protein accumulation depended on changes in RNA metabolism. It was concluded that cell division and net protein accumulation could occur without synthesis or accumulation of new ribosomes (Fraser, 1975b). In contrast, two periods of putative messenger RNA synthesis were identified, which were necessary for cultures to become competent to complete the following cell divisions (Fraser, 1975a).

The artichoke tuber tissue culture system has limitations if considered purely in the context of cell cycle studies. Excision and culture induce a process of de-differentiation, so that cells produced after each successive division become progressively more different from those at the start. However, the system does have merit for studying the processes involved when mature, differentiated, non-dividing storage parenchyma cells are induced synchronously to de-differentiate and revert to meristematic, actively-dividing cells.

Plant-virus interactions

In many branches of prokaryotic and eukaryotic biology, investigators have utilized viruses as useful model systems for study of the control of gene expression in the organism. Higher plants are no exception. Apart from dissecting how viral genes are expressed and controlled in the host cell, interesting questions are raised about the molecular mechanisms involved in the control of the host-virus

interaction. How is it, for example, that something as simple as tobacco mosaic virus (TMV), with a genome coding for only three or four proteins, can take over the immensely more complex host plant and virtually convert it into an assembly line producing virus particles? In a successful infection, some 75% of the host protein and RNA synthesizing capacity can be diverted to production of viral nucleoprotein (Fraser, 1973; Fraser and Gerwitz, 1980). By the end of multiplication the virus can amount to 10% of the total dry weight (Fraser, 1972).

The examination of host-virus interactions reported in this thesis originally concentrated on TMV infection of tobacco, as the virus is highly stable and easy to quantify (Fraser, 1971). Studies on host RNA metabolism and protein synthesis (Fraser, 1969; 1972; 1973; 1973a; Fraser and Gerwitz, 1980) gave information on how host macromolecular synthesis was altered, and suggested levels of control. Further work concentrated on plant growth regulators and gave some insight into their role in control of growth and metabolism in virus-infected plants (Whenham and Fraser, 1980; 1981; Fraser and Whenham, 1982).

Despite the comparative molecular simplicity of TMV - its molecular weight is only 40×10^6 - and the fact that it is one of the most intensively studied plant viruses, there are still areas where our understanding of it at a purely chemical level is incomplete. An early study (Fraser, 1973a) showed that although its RNA is a messenger (plus) sense strand, it does not contain the polyadenylic acid sequence characteristic of many eukaryotic (and viral) messengers. The same paper showed that TMV RNA contains no detectable (i.e. less than six) methylated sites on the bases or ribose moieties, a

finding consistent with messenger function. Subsequently, it has been shown that TMV RNA contains a 7-methyl guanosine 'cap' at the 5' end (Richards, Guilley, Jonard and Hirth, 1978). This low level of methylation would not have been detected by the technique used previously (Fraser, 1973a).

An interesting and provocative report was that TMV RNA contained between 5 and 17 modified nucleoside residues with cytokinin bioactivity (Sziraki and Balazs, 1979). However, re-examination of the question using a newly-developed and highly sensitive gas chromatographic assay for cytokinins (Whenham, 1981) as well as bioassay failed to reveal any cytokinins at detectable levels in TMV RNA (Whenham and Fraser, 1982).

Leaving aside their interest as model genes and probes for analyzing host biochemistry, plant viruses have obviously a more sinister side. They cause considerable damage and loss of yield in many crop species. At present no chemical treatments, analogous to fungicides for fungal diseases, are available for virus infections. Work on methyl benzimidazol-2yl carbamate (MBC), the fungitoxic principle and water-decomposition product of the benomyl fungicides (Clemons and Sisler, 1969) suggested that this compound could suppress formation of TMV mosaic symptoms in tobacco (Tomlinson, Faithfull and Ward, 1976). Further experiments reported in this thesis showed that this compound could strongly inhibit TMV accumulation; defined its effects on virus multiplication, symptoms and plant growth, and provided some indication of its possible mode of action (Fraser and Whenham 1978; 1978a). MBC may eventually receive approval for use against specific virus diseases of high value cash crops. However, any future development of chemotherapeutic agents is more likely to be based on a rational understanding

of the biochemistry of host-virus interaction rather than on the present somewhat empirical approach.

The method of choice in combatting virus disease is host resistance, if this is available in the particular crop. Many examples of resistance are known; they may be grouped conveniently into three types of mechanism.

1. 'Non-host' immunity occurs where a plant is not normally a host for a particular virus and is completely unaffected by it. For example, TMV will infect and multiply in tobacco, tomato and certain types of bean. In contrast, bean common mosaic virus (BCMV) will infect beans but has no effect on tobacco or tomato. The mechanism of their immunity is not understood, but one possibility is that BCMV replication is so finely attuned to particular features of the bean host that it fails in other hosts. This type of resistance is possibly non-researchable, and probably non-exploitable.

2. Constitutive, or genetically controlled resistance, is where a particular cultivar contains a gene or genes conferring resistance to a virus normally infecting that species. In this context resistance is defined as any inhibition of virus multiplication or pathogenic effects. Such resistances are known in many crops, and in many cases provide highly useful protection. The possibility of future, increased exploitation of constitutive resistance emphasises the importance of conservation of exisiting genetic resources by gene banking (Fraser, 1980).

Superficially at least, it appears that many, diverse mechanisms are involved in constitutive resistance, but in no case is there a full biochemical undeestanding of how resistance works. We are

therefore not in a position to exploit them fully in developing crop protection strategies, and spreading available resistance mechanisms to crops where at present no suitable resistance is available. This is the practical background to studies of resistance to TMV in tomato (Fraser and Loughlin, 1980; 1982; Fraser, Loughlin and Connor, 1980). This work has the long term aim of understanding the biochemistry of action of the Tm-1 resistance gene, and of defining the change in TMV which can allow it to become pathogenic on Tm-1-containing plants.

3. Induced resistance occurs when a plant normally susceptible to a particular virus has a resistance to that virus conferred by a prior infection or chemical treatment. Unlike constitutive resistance, induced resistance is not inherited, but must be induced afresh in each generation. It is interesting to contrast plants, where constitutive mechanisms predominate and have been widely exploited in breeding, with animals, where induced mechanisms such as the antibody and interferon systems are the major defences against virus infections.

Attempts have been made to find mechanisms parallel to the animal interferon system in plants (Sela, 1981) but the mechanism remains to be rigorously established and shown to have truly inducible antiviral activity. In related research, Kassanis, Gianinazzi and White (1974) proposed that the apparent resistance of tobacco plants to TMV infection which is induced by a previous, localized infection with the same virus, might involve certain host-coded 'pathogenesis-related' proteins which accumulate systemically after the first infection. However, closer analysis of the apparent resistance induced, and the involvement of 'pathogenesis-related' proteins, has questioned the effectiveness and usefulness of this

mechanism, and has suggested an alternative explanation of the phenomenon (Fraser, Loughlin and Whenham, 1979; Fraser, 1979; 1981; 1982). It is unlikely that this mechanism will find use in crop protection.

Fortunately, other experiments using systemically-spreading but highly attenuated strains of TMV have produced effective protection against subsequent infection by virulent strains (Fraser, 1981a). Current research is characterizing the molecular mechanisms involved, with the ultimate objective of using this knowledge and recombinant DNA techniques to develop novel types of virus resistance genes.

Isolated pieces of research, included in this thesis but not falling into the main subject areas discussed above, included development of techniques for detection and assay of polyadenylated messenger RNAs (Fraser and Loening, 1974) and for radioassay of the plant growth substance abscisic acid (Whenham and Fraser, 1981a). Aspects of RNA metabolism were studied in two developing systems: germinating S. pombe ascospores (Padilla, Creanor and Fraser, 1974) and germinating carrot and tobacco seeds (Brocklehurst and Fraser, 1980).

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(Reprinted from Nature, Vol. 244, No. 5413, pp. 222–224, July 27, 1973)

Rapid and Selective Inhibition of the Synthesis of High Molecular Weight RNA in Yeast by Lomofungin

LOMOFUNGIN (1-carbomethoxy-5-formyl-4,6,8-trihydroxyphenazine), an antibiotic from *Streptomyces lomodensis*, inhibits the growth of bacteria, yeasts and fungi[1,2]. It has been suggested that in *Saccharomyces cerevisiae*, the primary action of the antibiotic is an inhibition of RNA synthesis[2,3]. Cano, Kuo and Lampen[4] found that lomofungin inhibits three DNA-dependent RNA-polymerases extracted from *S. cerevisiae*. We have characterized the effects of lomofungin on RNA synthesis in the fission yeast, *Schizosaccharomyces pombe*.

S. pombe strain 132 was grown in a minimal medium, EMM 2 (ref. 5). Cells from exponential phase cultures were collected by centrifugation and resuspended in medium containing lomofungin. Control cells were resuspended in medium alone. Measurements of RNA synthesis were made during various periods after addition of lomofungin by supplying ^{32}P-phosphate, ^{3}H-adenine or ^{3}H-uridine.

Synthesis of ribosomal RNA (rRNA) and of polydisperse RNA was markedly inhibited within a few minutes of addition of lomofungin (Fig. 1*a*, *b*; Table 1). Pulse-labelling experiments carried out 15–35 min after addition of the antibiotic showed that the synthesis of polydisperse RNA, presumed to contain messenger RNA, had virtually ceased. Synthesis of rRNA was also almost completely inhibited (Fig. 1*c*; Table 1). Ribosomal and polydisperse RNA syntheses had very similar sensitivities to varying lomofungin concentrations (Table 2).

In cells pulse-labelled 1–6 min after the addition of lomofungin, the proportion in the precursor rRNA peak of the total radioactivity incorporated into all rRNAs (precursor[6] plus mature rRNAs) was greater than the proportion in precursor rRNA in similarly labelled control cells (Fig. 1*a*, *b*). This suggests that lomofungin not only inhibited the synthesis of

Table 1 Inhibition of RNA Synthesis after Addition of 60 μg ml^{-1} Lomofungin

Period of labelling (min)	Label	Percentage inhibition of incorporation into			
		rRNA*	Polydisperse RNA	5S RNA	tRNA
1–6	^{3}H-Adenine	57	53	—	—
15–30	^{3}H-Uridine	87	—	−5	−6
30–35	^{3}H-Adenine	95	97	6	34

The data were derived from gel scans such as those shown in Figs. 1 and 2. The total counts incorporated into an RNA species were found by adding the radioactivities of the individual gel slices comprising the radioactivity peak of that RNA, allowing for the background of polydisperse radioactivity in the gel. The inhibition of RNA synthesis by lomofungin treatment was found by comparison of incorporation in lomofungin-treated cells with control cells labelled in the same conditions.

*Includes the two mature species of rRNA and the rRNA precursors.

Table 2 Inhibition of RNA Synthesis by Various Concentrations of Lomofungin

Lomofungin (μg ml^{-1})	1	10	60
% Inhibition of rRNA*	63	73	94
Polydisperse RNA	62	65	81

Cells were labelled with ^{32}P-phosphate (Radiochemical Centre, Amersham) 10–30 min after adding lomofungin at various concentrations. Control cells were labelled without lomofungin. The total incorporation into rRNA and polydisperse RNA was measured from gel separations as explained in Table 1, and percentage inhibitions calculated by comparison with the control values.

*Includes the two mature species of rRNA and the rRNA precursors.

rRNA but also inhibited the processing of rRNA precursors to mature rRNAs.

In direct contrast to its strong inhibition of the synthesis of high molecular weight RNA, lomofungin did not inhibit the synthesis of low molecular weight RNAs. Figure 2 and Table 1 show that the synthesis of the 5S rRNA was completely unimpaired by lomofungin treatment even 30–35 min after addition of the drug. Transfer RNA (tRNA) synthesis was not inhibited 15–30 min after lomofungin addition, and slightly inhibited later (Table 1).

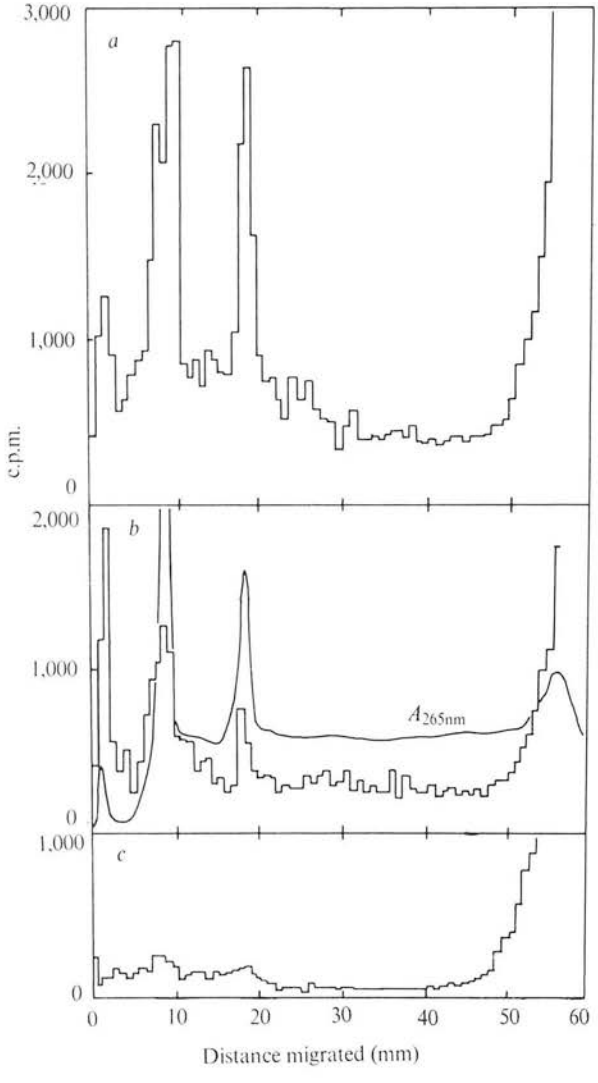

Fig. 1 Polyacrylamide gel electrophoresis of nucleic acids extracted from *S. pombe*. Cells were incubated with ^{3}H-adenine (specific activity 22.9 mCi mmol^{-1}; Radiochemical Centre, Amersham) at 25 μCi ml^{-1} for 5 min periods. *a*, Control; *b*, 1–6 min after addition of lomofungin (60 μg ml^{-1}); *c*, 30–35 min after addition of lomofungin (60 μg ml^{-1}). Cells were chilled at the end of the radioactive incubation, washed twice by centrifugation with ice-cold water and suspended in 2% tri-isopropylnaphthalene sulphonate; 0.1 M Tris-HCl, *p*H 9.0. The cells were broken in an Eaton press[5]. The homogenate was deproteinized by emulsifying once with 50% v/v chloroform, 50% v/v of the phenol/*m*-cresol mixture of Kirby[13] and twice with the phenol/*m*-cresol mixture. Nucleic acids were precipitated by making the aqueous phase 0.3 M NaCl and adding 2.5 volumes of ethanol. After three cycles of purification by reprecipitation[14], the nucleic acids were fractionated by electrophoresis on 2.6% polyacrylamide gels[15] for 2.5 h at 5 mA per gel, 8 V per cm gel length. Gels were scanned for absorbance at 265 nm in a Joyce-Loebl Gel Scanner, frozen in solid CO_2 and sliced transversely at 0.5 mm intervals with a Mickle Gel Slicer. Radioactivity in each gel slice was determined as described[16]. The continuous line shows absorbance at 265 nm; the histograms show radioactivity. The ultraviolet absorbance peaks are: at 10 and 19 mm, 25S and 18S rRNA; at 55 mm, tRNA. The radioactivity peak at 3 mm is the rRNA precursor.

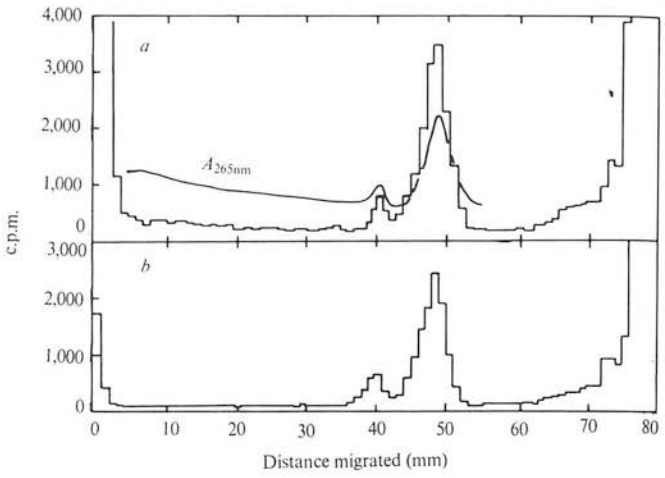

Fig. 2 Polyacrylamide gel electrophoresis of *S. pombe* RNA. Cells were incubated with 25 μCi ml^{-1} ^{3}H-adenine for 5 min (*a*, control) or 30–35 min after addition of 60 μg ml^{-1} lomofungin (*b*). Extraction of RNA and subsequent treatment were as described in Fig. 1. Electrophoresis was on 7.5% acrylamide gels for 2.5 h. The peaks on the ultraviolet absorbance scan (continuous line) are 5S RNA at 40 mm and tRNA at 49 mm. The radioactivity at 70–80 mm is free ATP.

It is possible that tRNA might have become labelled by turnover of its terminal CCA triplet[7] rather than by *de novo* synthesis, so masking any effect of lomofungin on tRNA synthesis. Labelling of tRNA by terminal triplet turnover when the isotope supplied was ^{3}H-uridine could only occur if the cell were actively synthesizing ^{3}H-CTP through amination of ^{3}H-uridine. To test this possibility, ^{3}H-uridine-labelled RNA from cells incubated with and without lomofungin was hydrolysed in 0.5 N NH_4OH; nucleotides were separated by high voltage electrophoresis[8] and radioactivity in each was determined. The radioactivity associated with the cytidylic acid ultraviolet absorbance marker spot was about 1% of the radioactivity in the uridylic acid spot. Therefore the possibility that significant labelling of tRNA by terminal triplet turnover occurred when ^{3}H-uridine was supplied is excluded.

The uninhibited synthesis of 5S and tRNA which occurred in lomofungin-treated cells at the same time as inhibition of rRNA and polydisperse RNA synthesis makes it unlikely that the inhibition of RNA synthesis by lomofungin could be indirect, that is by inhibition of some related aspect of cell metabolism, or by inhibition of the uptake of isotope. We were also able to exclude these explanations by direct experiment. Protein synthesis, measured by the incorporation of ^{3}H-leucine into trichloroacetic acid-precipitable material, showed a marked impairment only after 30–40 min of treatment by lomofungin at 60 μg ml^{-1}. The growth of cells, measured by the change in optical density of the culture, continued for 60 min after addition of 60 μg ml^{-1} lomofungin. Kuo, Cano and Lampen[9] found similar effects of lomofungin on protein synthesis and cell growth in *Saccharomyces cerevisiae*. The slow inhibition of protein synthesis and cell growth by lomofungin, compared with the rapid inhibition of RNA synthesis, suggests that lomofungin did not inhibit RNA synthesis indirectly by inhibiting some other aspect of cell metabolism such as energy supply. Lomofungin at 60 μg ml^{-1} did inhibit the uptake of ^{3}H-adenine by *S. pombe*, but only by 40% over the first 30 min after addition of lomofungin. This was much less than the inhibition of incorporation of ^{3}H-adenine into RNA (Table 1); therefore the measured inhibition of RNA synthesis was not a consequence of inhibited uptake of isotope.

We conclude that lomofungin selectively inhibits the synthesis of rRNA and polydisperse RNA, while having no effect on the synthesis of tRNA or 5S RNA. In comparison, Price and Penman[10] found that the synthesis of 5S and tRNA by isolated HeLa cell nuclei was not inhibited by α-amanitin,

whereas the synthesis of polydisperse RNA was. But α-amanitin does not inhibit rRNA synthesis[11].

Unlike actinomycin D (ref.12), lomofungin enters the yeast cells and inhibits RNA synthesis very rapidly. This antibiotic will prove of value in studies of the mechanisms of control of enzyme synthesis (refs. 2 and 9).

This work was supported by research grants from the MRC and SRC. We thank Drs Cano, Kuo and Lampen for allowing us to see their results before publication, and Dr George B. Whitfield of the Upjohn Company, Kalamazoo, Michigan, USA, for the gift of lomofungin.

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Received March 16, 1973.

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Printed in Great Britain by Flarepath Printers Ltd., St. Albans, Herts.

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CELL CYCLE CONTROLS

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New York San Francisco London

EARLY EVENTS IN THE GERMINATION OF *S. POMBE* ASCOSPORES

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I. Introduction

In the context of embryogenesis and organogenesis, cellular differentation is an extension of subcellular specialization.

Multicellularity probably evolved with the advent of the compartmentalization of the cytosol into discrete organelles. In fact, the physiological and metabolic diversity of differentiated cells is dependent not only on the maintenance of subcellular organelles but on the fidelity of their replication from cell division to cell division. It is at this level that genetic mechanisms must operate in directing the synthesis or renewal of enzymes, macromolecules and structures specific to individual subcellular organelles. In a diseased state such as oncogenesis, the loss of this controlling elements leads in no small measure to de-differentiation. This is one of the reasons why much attention is presently being directed towards achieving a greater understanding of the relationships between the repetitive events which comprise the cell cycle and those transitions which lead from one differentiated state to another. Such interactions are seen in developmental systems where differentiation and the cell cycle impinge upon one another. They are also found in the spore-cell transition in bacteria (1).

A similar sequence of events is seen in conjugation, sporogenesis and germination in yeast (2). This is a system in which subcellular differentiation and genetic control of the cell cycle come into direct interplay. The H90 sportulating haploid strain of *Schizosaccharomyces pombe* is particularly suited for these studies. It yields populations of spores in excess of 80%, which, as will be discussed in this communication, can be isolated in pure form and induced to germinate in a controlled manner. Unlike the budding yeasts, *S. pombe* possesses other advantages worth mentioning. It is haploid during most of its growth and cell cycle (3-5) and assumes a diploid level of DNA only as a zygote which is formed following conjugation between two vegetative cells. Two meiotic divisions ensue usually resulting (6,7) in the formation of four ascospores per ascus. The spores are spontaneously liberated and may be stored indefinitely in a freezer. When placed in a nutrient medium, they readily germinate and proceed into vegetative cell growth and division.

We thus have a well delineated sequence of events and complex biological

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processes which are separated in time and may be subjected to experimental manipulation. The synchronization of any one of these phases would be of considerable practical significance. It would allow one to study the underlying controlling mechanisms (e.g., sequential enzyme synthesis or induction, genomic readout, etc.) during the transition from one state to another.

In the present report, we have focused our attention on the early events of germination and will describe the experimental approaches used to study the initial macromolecular events underlying that process.

II. Experimental

Vegetative cells of *S. pombe* (H90 strain, NCYC 132) were grown in the chemically defined EMM 2 medium or malt extract broth (4). Sporulation was induced by plating cells from a stationary or late-log culture onto the surface of 2% agar (w/v) containing 1.5% malt extract broth (Oxoid), and incubating at 32° for 7-14 days. The spores and cells were harvested by washing the agar surface with distilled water. They were freed of debris by two centrifugations (5 min, 1500 RPM) in a bench top centrifuge. The pelleted cells and spores were stored frozen at -20° until needed.

Although this strain will routinely yield large quantities of spores (80±5%), they cannot be reproducibly freed from the residual vegetative cells by a single centrifugation through linear sucrose density gradients using conventional low speed swinging bucket centrifuges (3). Such a method of separation was developed by Mitchison and coworkers to select highly synchronous populations of vegetative *S. pombe* cells (8,9). In addition, it was observed that the individual spores were highly heterogenous with respect to size and onset of germination (Mitchison, personal communication and 10). We thus investigated alternative methods of spore isolation, the most successful approach being the use of the analogue, 2-deoxyglucose, to produce not only "weak walled" spores, but to differentially induce lysis of the residual vegatative cells as shown by previous investigators (11,12).

The time course of germination was followed by time lapse photomicroscopy of spores that were resuspended in EMM 2 and spread over small agar pads placed in a wax-sealed coverslip (13). The coverslip preparations were incubated at 32°. It was thus possible to follow the development of spores whose dimensions were determined from enlarged photographs or projections. Parallel sets of spores were also resuspended in liquid media and placed in a water bath set to reach 32° by a delayed time device (4). Techniques for isotopic labelling, chemical analyses, etc., used with *S. pombe* have been summarized by Mitchison (4).

A. Size Distribution of Ascospores

In a recent study, Yoo et al (14) found that the onset of ascosporogenesis in a homothallic haploid strain of *S. pombe* was asynchronous, even for spores within the same ascus. Spores reached different levels of "maturity" even

though they presumably arose from the same zygote. In addition, spores were formed by having unequal quantities of cytoplasm enclosed by the enveloping "spore membranes." Thus spores of different sizes were seen within the same ascus. If conjugation itself is asynchronous, there would be little reason to expect that the ensuing germinative events would be synchronous, unless spores could be selected into narrow size or age classes. The biological significance of asynchrony of ascosporogenesis is unknown. Asynchrony may simply reflect the randomness usually associated with growing populations of cells, or it may be part of a mechanism designed to prevent self-mating between spores released from the same ascus. In any event, it became necessary to determine the extent of the heterogeneity in the H90 spores and to see how it would affect the timing of germination.

Newly formed spores were harvested from the agar surface as previously described. Aliquots were diluted with formalin saline (0.9% NaCl, 1.0% formalin) to a density of approximately 10^5 cells/ml to minimize coincident loss of electronic counting. The spores were dispersed by sonication (3,4) and counted with a Coulter counter (Model B, 70 μm aperture). Parallel counts were taken with a hemocytometer.

The counter was calibrated by measuring the size distribution of latex spheres of known diameters (7.6±2.3 μm, Dow Chemical Corp., Diagnostic Products, Indianapolis, Ind.) and spores that were isolated into distinct size classes by zonal density centrifugation (10). The diameters of the spores were measured from enlarged photographs taken under dark-field phase microscopy (4,13). (The Coulter counter had a resolving power of from 1-2.5 μm^3/threshold unit, depending on the settings used.)

The size distribution of a mixture of spores and cells is shown in Fig. 1. The size distribution of spores found in the presence of 2-deoxyglucose is also shown. (This part of the experiment will be discussed later.) The median volume of the control cells is 37 μm^3. It is clear that the size distribution is bimodal. The distribution to the left is presumably generated by the spores. The range of volumes is from about 20 to 90 μm^3. This is in good agreement with the range of spore sizes determined photographically (17.8-93.9 μm^3, 10). The smaller distribution to the right is probably generated by the residual vegetative cells that failed to conjugate and sporulate. As shown by Mitchison (15), vegetative cells of *S. pombe* had an average volume of 140±22 μm^3 at the time of cell plate formation. Considering that this value was derived from cells in exponential growth and under different culture conditions than used in this study, the agreement with our determination is good. It falls well within the volume range of ca. 100-220 μm^3 shown in Figure 1. Measurement of the relative proportions of these two distributions by planimetry showed that the smaller distribution is 14.7% of the total distribution. This is the same as the proportion of residual vegetative cells (see Table 1).

The lack of overlap between the size distributions of the two populations suggested that they could be easily separated by density gradient centrifugation. Indeed, zonal density centrifugation in the A-XII rotor system

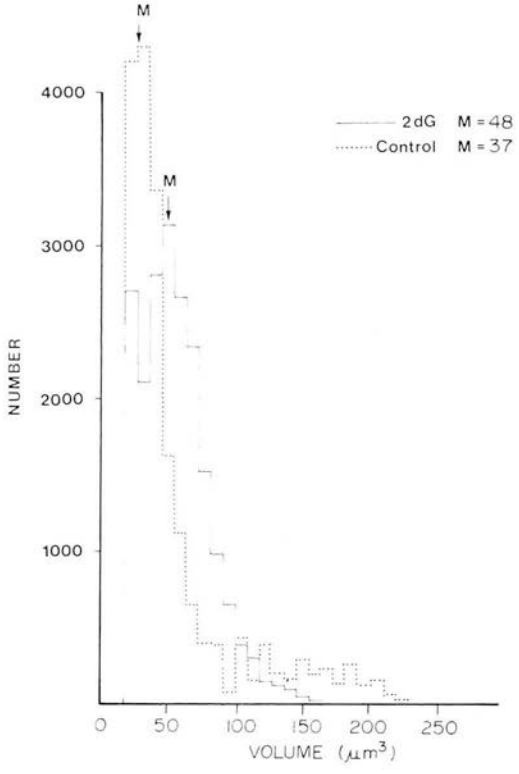

Fig. 1. Size distribution of *S. pombe* ascospores formed in the presence and absence of 2-deoxyglucose. Sizing was done with a Coulter counter as described in the text. The median volumes are indicated by the arrows.

(16, 17) was subsequently employed to separate the spores into distinct size classes (10). As mentioned before, we used an alternate approach to eliminate the vegetative cells. We added 2-deoxyglucose to malt extract agar to induce lysis of the residual vegatative cells.

This effect is illustrated by Fig. 1 and Table 1. *S. pombe* cells taken from liquid cultures in late log or early stationary phase of growth were plated onto malt extract broth agar (slants containing varying concentrations of 2-deoxyglucose added just before autoclaving). After 7-15 days, the cells were harvested and analyzed microscopically. They were also sized with the Coulter counter as previously described. It is clear that 2-DG had at least two concentration dependent effects: (a) At concentrations between 20 and 50 μg/ml, it reduced the percentage of residual vegetative cells to one-fifth of that of the control cells (Table 1). Fig. 1 shows this effect for cells exposed to 40 μg/ml. In terms of size distribution, there were very few cells with volume

TABLE 1

Effect of 2-Deoxyglucose on Sporogenesis of *S. pombe*

Concentration (μg/ml)	Spores	Asci (Percent)	Vegetative Cells
0	78.8	1.3	19.9
20	92.2	0.6	7.2
40	95.9	0.8	3.3
50	94.3	-	5.7
77	68.0	16.0	16.0
80	20.8	7.1	72.1
154	16.0	25.0	59.0

greater than 100 μm^3. A secondary effect was also evident, the median volume increased to 48 μm^3. The spores reared in the presence of 2-deoxyglucose are larger than control spores, but as will be discussed later, will germinate later. (b) At concentrations above 50 μg/ml, 2-deoxyglucose interfered with the process of ascosporogenesis. The spore yield dropped considerably, the proportion of asci, many of which contained poorly matured spores, rose and the number of residual vegetative cells increased sharply. They were enlarged, misshapen, and many of them had multiple cell plates. In addition, they were easily lysed during the washing procedure in harvesting. Because of this, in all subsequent work we used concentrations of 2-DG of 40 μg/ml.

B. Kinetics of Germination

The results described above raised the question as to the effect of 2-deoxyglucose on the kinetics of germination. An experiment was performed in which *S. pombe* cells were allowed to conjugate and sporulate in the presence of 2-deoxyglucose (40 μg/ml). The spores were harvested and analyzed by time-lapse photomicrography as previously described. Care was taken, at harvest time, to assure that the spores were freed of residual medium. They were resuspended in EMM 2 and allowed to germinate on small agar pads (2% agar in EMM 2) using the coverslip preparation (13). They thus germinated in the *absence* of 2-deoxyglucose. Table 2 summarizes the data from this experiment. It shows the relationship between the initial volume (calculated from diameters measured on enlarged photographs) and time at which the onset of outgrowth and cell plate formation occurred. Outgrowth is defined as the time when spores become markedly elliptical ("pear shaped"). Photographs were taken at 30 minute intervals.

Although the number of spores analyzed in this experiment was small (2-DG, n=39; control, n=37), the data show that larger spores germinated earlier. This is in agreement with a prallel study in which a larger sample was analyzed (10). 2-DG introduced approximately a two hour delay in the onset of outgrowth (i.e., for spores of the same size). The time interval between the

TABLE 2

Kinetics of Germination of *S. pombe* spores as a Function of Volume and Sporogenesis in the Presence of 2-Deoxyglucose (2DG)

Sporulation Conditions	Volume (μm^3)	Outgrowth (hrs ± SD)	Division (hrs ± SD)
CONTROL	40	6.8 ± 0.8	11.3 ± 1.0
	63	6.4 ± 0.6	10.4 ± 0.6
	94	5.2 ± 0.6	9.3 ± 0.9
2-DG (40 μg/ml)	40	8.2 ± 0.5	12.7 ± 0.2
	50	7.6 ± 0.9	11.9 ± 1.5
	63	7.0 ± 1.0	11.3 ± 1.0

onset of outgrowth and the first cell division (i.e., cell plate formation) remained constant in both situations. It is about 4.5 hours long.

The kinetics of germination were further analyzed on a larger sample of ascospores separated into a narrower size class by centrifugation through a 15-40% linear sucrose density gradient in an A-XII zonal rotor as described elsewhere (16,17). The spores were harvested as described above and allowed to germinate on EMM 2 agar pads. (Fig. 2) The time course of germination is expressed as the germination index (No outgrown/total number x 100). It was determined on at least 150 spores per point. The mean and standard deviation of the volume of the 2-DG spores was 33.0±0.02 μm^3 and that of the control was 31.5±0.02 μm^3. It is clear that the control spores germinated much more rapidly than the 2-DG reared ones, even though the latter began to outgrow earlier. The time taken for the germination index to rise from 10 to 90% was 5 hours for the 2-DG spores and 3 hours for the control ones. Although the onset of the first vegetative division was not measured, it presumably would follow similar kinetics as shown in Table 2.

Notwithstanding the fact that control spores germinate more "synchronously," they were not as "fragile" as the 2-DG spores. For example, preliminary experiments showed that in order to achieve a breakage in excess of 75%, the control spores had to be subjected to repeated passes through an Eaton Press (which is a simplified version of the French Press). On the other hand, over 90% of the 2-DG spores were broken within 3 minutes with a Vibromixer (Shandon Scientific, London). This instrument vibrates a fluted stainless steel probe dipping into a slurry consisting of 5 ml containing $9.6x10^8$ cells and 4 gms of glass beads (0.2 mm in diameter). This provides a rapid and convenient method of breaking up spores and was the method used in all subsequent experiments for the extraction of the RNA. All the experiments to be described below were performed on spores which were formed in the presence of 40 μg/ml 2-DG for this reason.

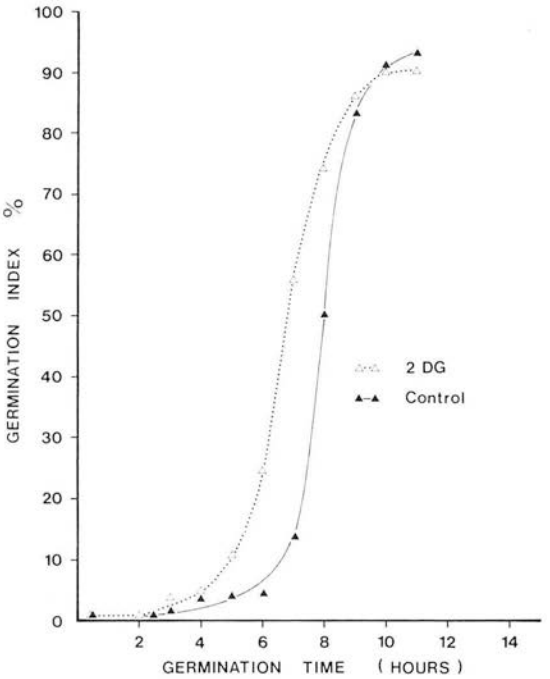

Fig. 2. Kinetics of germination of *S. pombe* ascospores that had sporulated in the presence and absence of 2-deoxyglucose. Spores were separated by zonal density centrifugation. Germination was at 32° on EMM 2 agar pads as described in the text.

C. Early Macromolecular Events During Germination

1. Protein and RNA Synthesis. Synthesis of RNA and protein, measured by the incorporation of labeled precursors into TCA-precipitable material, began within the first hour of germination (Fig. 3). In this experiment, spores were resuspended in EMM 2 to which the appropriate precursors were added. The kinetics of germination of these spores are shown in the insert. It is clear that even though that outgrowth did not begin until the 3rd hour and a 50% index was not reached until the 8th hour, incorporation of both labels began within the first 30 minutes of germination. In a parallel study, we found that the relative *rate* of uptake of ^{3}H-adenine increased at a linear rate with doubling in rate by the 5th hour of germination.

In order to determine which species of RNA were being synthesized during this interval, we isolated the RNA of spores that had been exposed to a 20-minute pulse of ^{3}H-adenine at 30, 180 and 300 minutes after being resuspended in EMM 2. The pelleted spores were then broken by the

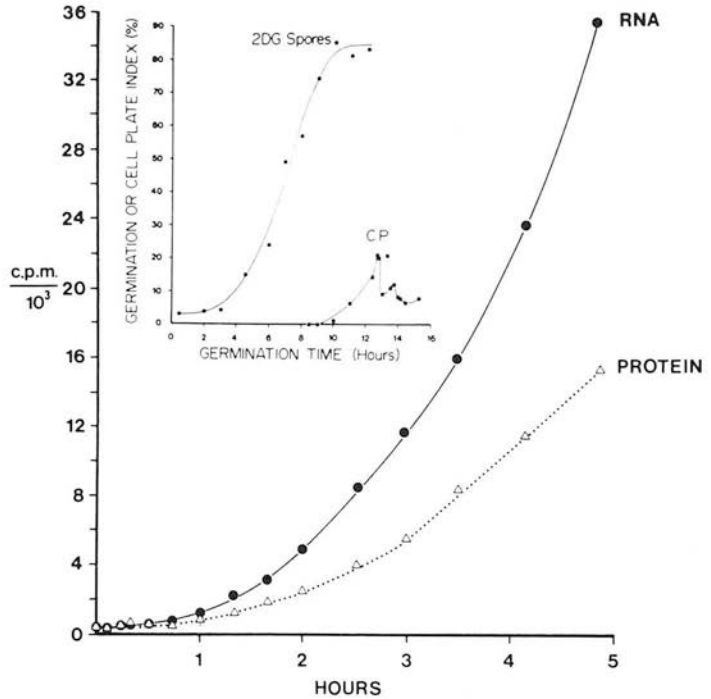

Fig. 3. Incorporation of ^{3}H-leucine and ^{3}H-adenine into trichloroacetic acid precipitable material of *S. pombe* spores continuously exposed to the label in EMM 2. The kinetics of germination are shown in the insert. *Insert.* Kinetics of germination of spores formed in the presence of 2-deoxyglucose and separated by centrifugation through a sucrose density gradient. The germination and cell plate indices (ordinate) were determined photographically as described in the text.

Vibromixer and nucleic acids were extracted by a detergents-phenol procedure (21). The DNA was not removed. The RNA was fractionated on 3.0% polyacrylamide gels (22) for 3 hours at 8 V/cm; 5 mA/gel. The gels were scanned for ultraviolet absorption at 265 nm with a Joyce-Loebl Gel Scanner. The gels were frozen in solid CO^2 and sliced transversely at 0.8 mm intervals with a Mickle Gel Slicer, incubated with NH_4OH, mixed with scintillation fluid and counted on a Packard spectrometer (18). Fig. 4 shows the results of these analyses. The specific radioactivity is expressed as counts per minute/unit of ribosomal RNA. The quantity of rRNA on the gel was calculated from the peak areas of 18 and 26S rRNAs on the ultraviolet absorbance scan (23). Note the change in the scale in each panel.

Thirty minutes after resuspension in EMM 2, the spores had begun to synthesize RNA of all sizes. The 26 S and 18 S rRNA species had specific

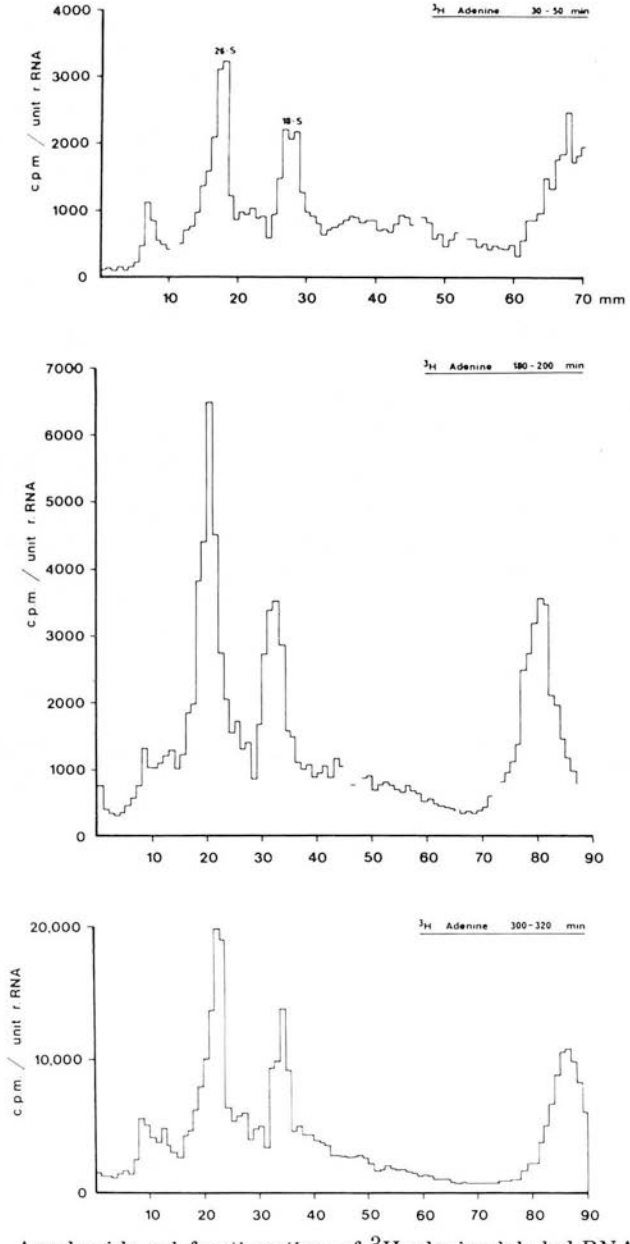

Fig. 4. Acrylamide gel fractionation of ^{3}H-adenine labeled RNA isolated from *S. pombe* spores. Spores were resuspended at t=0 in EMM 2 and exposed to 100 μCi, ^{3}H-adenine and 1 μg cold adenine/ml for 20 minutes. Top panel t=30-50 min; middle panel, t=180-200 min; bottom panel, t=300-320 min. The ordinate gives the specific activity as counts per minute/unit ribosomal RNA.

activities in excess of $3x10^3$ and $2x10^3$ cpm/unit rRNA, respectively. At 180 minutes, the specific activities had doubled and by 300 minutes of germination they had essentially tripled. Note that the spores were synthesizing proportionately equal molar quantities of these two species of RNA, suggesting that the synthesis is coordinated and that complete ribosomes were being made. In addition, polydisperse and high molecular weight RNAs were being actively synthesized.

2. *Messenger RNA Synthesis.* The labeling of polydisperse RNA suggested that messenger RNAs were being synthesized soon after the onset of germination. It has been reported that in budding yeast, some messenger RNAs contain a polyadenylic acid [poly(A)] sequence (24) and we have found that up to half of the polydisperse RNA of vegetative cells of *S. pombe* contains a poly(A) sequence (Fraser and Creanor, unpublished). We, therefore, looked for poly(A) sequences in the RNA of germinating spores. We used the technique developed by Fraser and Loening (18) to assay for the presence of messenger RNAs which contain poly(A) sequences. This technique involves hybridization of ^{3}H-polyuridylic acid [poly(U)] to the poly(A) sequences. The hybrids may then be fractionated, and freed from excess poly(U) by sedimentation on sucrose gradients, or the excess poly(U) may be removed by RNAs, the hybrid co-precipitated with carrier RNA and counted on glass fiber filters. The method avoids the need to label mRNA directly, and can be used to assay the total poly(A)-containing messenger RNA content of the cell, including pre-existing, stable messengers.

Approximately $2x10^8$ spores, sporulated in the presence of 2-DG, were allowed to germinate in 15 ml of EMM 2 at 32°. At various times, 5 ml of spores were collected and the RNA was extracted as described by Fraser *et al* (21). A portion of the RNA was fractionated through polyacrylamide gels to quantitate the amount of RNA present. The remainder of the RNA was mixed with an excess of tritium labeled polyuridylic acid (0.01 µg/ml, 0.2 µCi/µg, Miles Laboratories, Inc.) dissolved in 0.5% sodium dodecylsulfate, 150 mM NaCl; 1 mM EDTA; 50 mM Tris-HCl, pH 7.6. The SDS was removed by precipitation with 50µl M KCl (18). The supernate was digested with 10µg RNase/ml for 10 minutes at room temperature. Exactly 100µg of carrier RNA were then added and the mixture was treated with 3 volumes of 95% ethanol at -20° for 2 hours to precipitate the hybrids. They were collected on 20 mm glass filters (Whatman, GFA) and washed 3 times with 95% ethanol. The filters were dried and placed in scintillation fluid and counted with a Packard Tri-Carb scintillation spectrometer (18).

Figure 5 shows the results of this experiment. The ordinate gives the relative quantities of polyadenylic acid sequences per unit ribosomal RNA in each sample. It is clear that there was a three-fold increase in the relative amount of adenylate-rich RNA species within 30 minutes of germination. It is also apparent that spores that had not yet germinated had measurable quantities of these RNAs (t=0). When RNA was fractionated on a sucrose gradient, and each fraction was challenged with ^{3}H-poly (U) and treated with RNase as above, the

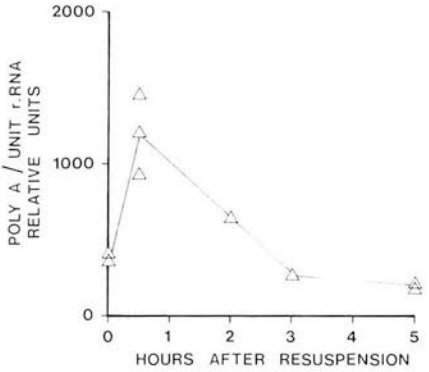

Fig. 5. Changes in the relative concentration of RNA containing polyadenylic residues as a function of germination. Spores were resuspended in EMM 2 at t=0, the RNA was extracted and hybridized with ^{3}H-labeled polyuridylic acid. See text for details.

sizes of those RNAs containing poly(A) sequences was shown by the distribution of radioactivity on the gradient. We found that poly(A) was broadly distributed from about 5 S to 30 S. This means that poly(U) hybridization was not measuring free poly(A), which is much smaller (24), but probably poly(A) sequences in messengers RNAs. In particular, poly(A) present at zero time was not a pool of free poly(A), but was in high molecular weight RNAs. Since the quantities of the mRNAs were determined in terms of the rRNA present, and since the latter was being synthesized rapidly at this time, there was a drop in the ratio of mRNA to rRNA between 30 minutes and 3 hours of germination. We interpret the results to show that the rate of rRNA synthesis was high at the start of germination. The rate of rRNA accumulation then increased faster than the rate of increase of mRNA content. Thus one of the first events in the germination process is the active synthesis of mRNA. (Please note that the method of poly(U) doesn't itself measure mRNA synthesis only cell content of mRNA. So the decline in the poly(U)/rNA data does not necessarily mean that the rate of mRNA synthesis is declining, only that the rate of rRNA accumulation is probably increasing).

3. DNA Synthesis. As shown by Bostock (3), the quantity of DNA in the spores of *S. pombe* is at the haploid level (ca 0.0146 picograms per spore). The time course of DNA synthesis during germination is not known. We tried to measure the bulk quantity of DNA but were unable to obtain a consistent value, largely because of the technical difficulties in the quantitative breakage of the spores. We measured, instead, the kinetics of incorporation of ^{32}P into the DNA at various times during germination.

A one milliliter aliquot (containing $2x10^{8}$ spores) of the same 2-DG spores used above were resuspended in 20 ml of EMM 2. At different times, 2 ml were removed and pulsed with 100 μCi of $^{32}PO_4$ (Radiochemical Centre,

Amersham, U.K., specific activity 1-9 mCi/ml) for 30 minutes. The tubes were rapidly chilled, centrifuged and the supernatant discarded. The spores were then broken with the Vibromixer and the nucleic acids were extracted as described above. The samples were treated with RNase (40μg/ml, 20 minutes at room temperature) and fractionated through 3% acrylamide gels. Slices of gels containing ^{32}P were dried and counted in a Geiger tube as previously described (18). Total ^{32}P incorporation into DNA was determined by addition of counts in slices making up the DNA radioactivity peak. The results are shown in Fig. 6.

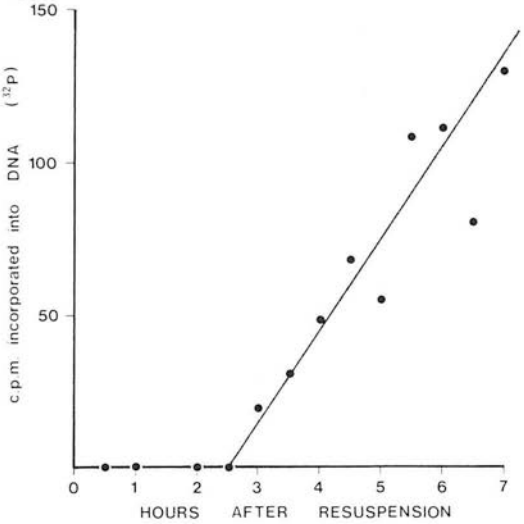

Fig. 6. Incorporation of ^{32}P-orthophosphate into the DNA of *S. pombe* spores following resuspension in EMM 2. See text for details.

There was no incorporation of ^{32}P into the DNA in the first 2.5 hours of germination. From that time the incorporation was linear, although the scatter in the points makes it impossible to state this with certainty. In any event, it is clear that at the outset, the spores were not engaged in any measurable synthesis of DNA. This would suggest that the onset of DNA synthesis must await the synthesis of a considerable quantity of RNA, and possibly also enzymes required for DNA synthesis. This is at best only a guess at this time.

III. Summary and Concluding Remarks

Germination of *S. pombe* spores can be separated into distinct macromolecular and cytological phases of activity. Upon resuspension in a nutrient medium, protein and RNA synthesis are initiated almost immediately and continue for several hours with a constantly increasing rate. Although we cannot detail the diversity of the proteins being synthesized, we do know a little more about the types of RNAs that are made. It seems that ribosomal RNA synthesis is coordinated so that both species of rRNA (26 S and 18 S) are

made concurrently. Secondly, although the spores have measurable quantities of putative messenger RNAs (i.e., RNA species rich in adenylic acid residues), there is a rapid increase in synthesis of this kind of RNA within the first 30 minutes of germination. The biological implications are obvious, the spore has a store of information sufficient for a "start in life."

As for the DNA, it would appear that there is a presynthetic period of approximately 2.5 hours duration, as indicated by the lack of incorporation of ^{32}P into the DNA. DNA synthesis then continues in a linear fashion up to the time of the first vegetative division. Thus, there is probably no G_2, spores are "G_1 types."

Cytologically the onset of outgrowth seems to be a function of initial spore size, while the interval between outgrowth and the first vegetative division is constant. This would imply that spores must achieve a minimal level of cellular growth before they can proceed towards cell division. What this implies in terms of subcellular replication is unknown, since we do not know exactly to what extent organelles such as mitochondria, Golgi, etc. were disassembled within the spore.

We have also shown that 2-deoxyglucose can be used to achieve the separation of spores from the residual vegetative cells. A dividend is the formation of spores that are much more easily ruptured. There is, however, an indication that a delay is introduced into the timing of the onset of outgrowth by 2-DG and possibly also a loss of synchronization. This secondary effect, may however, be artifactual in that the separation of spores on the basis of size alone may not be sufficiently precise to yield a high degree of synchrony of germination.

The conjugation-sporulation-germination-division sequence is of biological significance in itself. It offers us an opportunity not merely to follow complex changes in state, but to manipulate the reproductive events and thus alter the subsequent expression of the genetic complement. With the advent of specific mutants in this strain of yeast, it will be possible to use crossbreeding techniques in conjunction with macromolecular methods to pin point the controlling mechanisms in the expression of a spore's genetic complement.

Acknowledgements

This work was supported by the Medical Research Council of the U.K. and the Food and Drug Administration of the U.S.A. (Grant no. 5 RO1 FD-00120 to GMP). The senior author was a recipient of a Postdoctoral Research Fellowship from the Medical Research Council while on a sabbatical leave of absence in Professor Mitchison's laboratory. We also wish to thank Dr. B. L. A. Carter for his assitance in the zonal centrifugation and Miss Anne Baker for excellent technical assistance.

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Eur. J. Biochem. *46*, 67—73 (1974)

Rapid and Selective Inhibition of RNA Synthesis in Yeast by 8-Hydroxyquinoline

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(Received February 12/March 29, 1974)

A selective inhibitor of RNA synthesis is an essential tool for studies of the mechanisms of control of enzyme synthesis. Yeast is a particularly suitable organism for such studies, but until recently no suitable inhibitor of RNA synthesis has been available. 8-Hydroxyquinoline, at concentrations of 10—50 μg/ml, rapidly and selectively inhibits RNA synthesis in fission yeast. Protein synthesis, cell growth and uridine uptake are not immediately affected. The mechanism of inhibition appears to be by chelation of divalent cations required for RNA synthesis. An explanation of the selectivity of inhibition is proposed. An estimate of 20 min for the upper limit of messenger RNA half-life was obtained by following the rate of protein synthesis after inhibition of RNA synthesis by 8-hydroxyquinoline. At low concentrations, 8-hydroxyquinoline inhibits the synthesis of ribosomal RNA and messenger RNA, but has no effect on the synthesis of transfer RNA or 5-S RNA. This differential effect could arise from differing divalent cation requirements of different RNA polymerases.

Yeasts have been used for studies of the mechanisms of control of enzyme synthesis in eukaryotes because they are among the simplest eukaryotes, are easy to grow and synchronise, and genetic manipulation is feasible. However, a major drawback to their use has been the lack, until recently, of an inhibitor of RNA synthesis, an essential tool for studies of enzyme synthesis. Many known inhibitors seem to penetrate the cells poorly or fail to inhibit [1—4], although Tønnesen and Friesen [5] found good inhibition of RNA synthesis in *Saccharomyces cerevisiae* by extremely high concentrations of daunomycin or ethidium bromide. The phenazine antibiotic lomofungin has been shown to inhibit RNA synthesis in yeast at low concentrations [6—8]. During further studies on lomofungin, we found that its mode of action was chelation of divalent cations required for the activity of RNA polymerase (Fraser and Creanor, unpublished results). We therefore examined some other chelating agents as inhibitors of RNA synthesis. In this paper, we describe the rapid and selective inhibition of high-molecular-weight RNA synthesis in fission yeast by 8-hydroxyquinoline. We suggest how it can be selective for RNA synthesis without inhibiting other processes, such as protein synthesis, which also require divalent cations.

Abbreviations. Butyl-PBD 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole; poly(A), poly(adenylic acid); oligo(dT) oligo(deoxythymidylic acid).

Enzymes. DNA-dependent RNA polymerase or RNA nucleotidyl transferase (EC 2.7.7.6).

MATERIALS AND METHODS

Fission yeast (*Schizosaccharomyces pombe*) strain 132 was grown in a minimal medium EMM 2 [9]. Cell growth was followed by measuring the absorbance of cultures at 595 nm. Cultures in the early part of the exponential growth phase were used for experiments. The cell density was $2.5—4.0 \times 10^6$ cells/ml.

8-Hydroxyquinoline was dissolved at 1% (w/v) in 10% (v/v) acetic acid, and was added to cultures to final concentrations of 10—500 μg/ml. An equivalent volume of 10% acetic acid was added to control cultures. The slight pH change this caused in the medium made no difference to the rate of RNA synthesis.

The rate of total RNA synthesis was measured by the incorporation of [5-^{3}H]uridine into trichloroacetic acid-insoluble material. Isotope was added to the culture to a concentration of 20 μCi/ml. 0.5-ml samples of culture were mixed with 5 ml ice-cold trichloroacetic acid; the cells were collected by filtration on Whatman GF/A glass fibre paper and washed three times with 5 ml cold 5% trichloroacetic acid. Acid-insoluble radioactivity on the dried filters was measured by

counting in 0.5% (w/v) butyl-PBD-toluene scintillator in a Packard liquid scintillation spectrometer.

Total [^{3}H]uridine uptake was measured by mixing 0.5-ml samples of culture with 5 ml ice-cold water containing 100 μg/ml non-radioactive uridine. Cells were collected by filtration, washed three times with cold water and counted as above.

Protein synthesis was measured by the incorporation of [4,5-^{3}H]leucine into cold acid-insoluble material. Isotope was added to cultures to 20 μCi/ml, together with 20 μg/ml non-radioactive leucine. Incorporation was measured in the same way as uridine incorporation. Cold trichloroacetic acid also precipitates tRNA charged with [^{3}H]leucine. Control experiments, in which [^{3}H]leucinyl-tRNA was removed from the insoluble fraction by heating at 90 °C for 15 min [10] showed that [^{3}H]leucine tRNA did not contribute significantly to the total cold acid-precipitable radioactivity, which could therefore be taken as a measure of the [^{3}H]leucine incorporation into protein.

Certain aspects of nucleic-acid synthesis were examined in more detail. Cells were incubated with 100 μCi/ml [5-^{3}H]uridine or [8-^{3}H]adenine from 10 to 20 min after addition of 8-hydroxyquinoline. Total nucleic acids were extracted by a phenol-detergent procedure and further purified as explained previously [7].

To examine the effects of 8-hydroxyquinoline on the synthesis of different types of RNA, uridine-labelled total nucleic acid was fractionated by electrophoresis on polyacrylamide gels [10]. The gels were 2.4% acrylamide for the first 55 mm of their length and 10% acrylamide for the remaining 45 mm. Electrophoresis was for 3.5 h at 50 V. These composite concentration gels were used to permit fractionation over a wider range of molecular weight of RNA. The gels were scanned for absorbance at 265 nm and the distribution of radioactivity was determined as described previously [7].

To measure the synthesis of those mRNAs contained a poly(A) sequence [12], uridine-labelled total nucleic acid was dissolved in binding buffer [10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.1% (w/v) sodium dodecylsulphate, 400 mM NaCl]. Poly(A)-containing mRNAs were bound to oligo(dT)-cellulose (Collaborative Research Inc., Waltham, Mass.) [13] by shaking 100 μg RNA with 20 mg cellulose in 0.5 ml binding buffer for 30 min at 20 °C. The cellulose was washed five times with 0.5 ml binding buffer to remove non-specifically bound RNA. After each wash the cellulose was sedimented by centrifugation at 12000×*g* for 2 min. Poly(A)-containing mRNA was released by washing the cellulose three times with 0.5 ml binding buffer lacking NaCl. Carrier RNA was added to the combined messenger fractions and RNA was precipitated by adding trichloroacetic acid to 5%. The precipitate was collected by filtration on Whatman GF/A paper and counted as above.

As a check on the nature of the messenger fraction prepared by oligo(dT)-cellulose, RNA was precipitated from the combined messenger fractions prepared from control cells, by adding 100 μg non-radioactive yeast total RNA and 2 volumes ethanol. The RNA was analysed by electrophoresis on 3% polyacrylamide gels for 2.5 h. The radioactivity was found to be polydisperse, ranging from 5 to 30 S, with a mean around 15 S. There was very little contamination of the messenger fraction by labelled rRNA or tRNA.

For the determination of DNA synthesis, [^{3}H]-adenine-labelled nucleic acids were dissolved in 0.15 M NaCl; 0.015 M trisodium citrate pH 7.4 and RNA was digested with 20 μg/ml pancreatic ribonuclease for 20 min at 20 °C. DNA was separated from the digest by electrophoresis on a 2.4% polyacrylamide gel [11] for 2 h at 50 V. The sharp ultraviolet absorbance peak of DNA was detected by scanning the gel at 265 nm. The gel was frozen and sliced [7, 11] and DNA was hydrolysed out of the slices making up the DNA peak by incubating with 0.1 N HCl at 60 °C for 12 h. The nucleotides were dissolved in 0.3 ml water and counted in 7 ml water-miscible scintillator [0.5% (w/v) butyl-PBD, 40% (v/v) 2-methoxy-ethanol, 60% (v/v) toluene].

Mouse L-cells were maintained in suspension culture in Glasgow modified minimum essential medium (Biocult Ltd, Glasgow, Scotland) containing 5% (v/v) foetal bovine serum. The cell concentration was 10^5 to 10^6 cells/ml. Subculturing was at 5-day intervals. Cells were collected by centrifugation for 5 min at 500×*g* and resuspended in medium lacking divalent cations, and containing various concentrations of 8-hydroxyquinoline. RNA synthesis was measured by the incorporation of [5-^{3}H]uridine, protein synthesis by the incorporation of [4,5-^{3}H]-leucine. Samples of labelled cultures were collected on Whatman GF/A filters, washed three times with ice-cold Hank's balanced salts solution, then three times with 5% trichloroacetic acid and counted as above.

RESULTS AND DISCUSSION

Effects of 8-Hydroxyquinoline

Total RNA synthesis was inhibited immediately after addition of 8-hydroxyquinoline to yeast cultures (Fig. 1 A). Inhibition was strong at concentrations as low as 10 μg/ml and was complete at 100 μg/ml. The uptake of [^{3}H]uridine was inhibited by 8-hydroxyquinoline, but very much less than incorporation

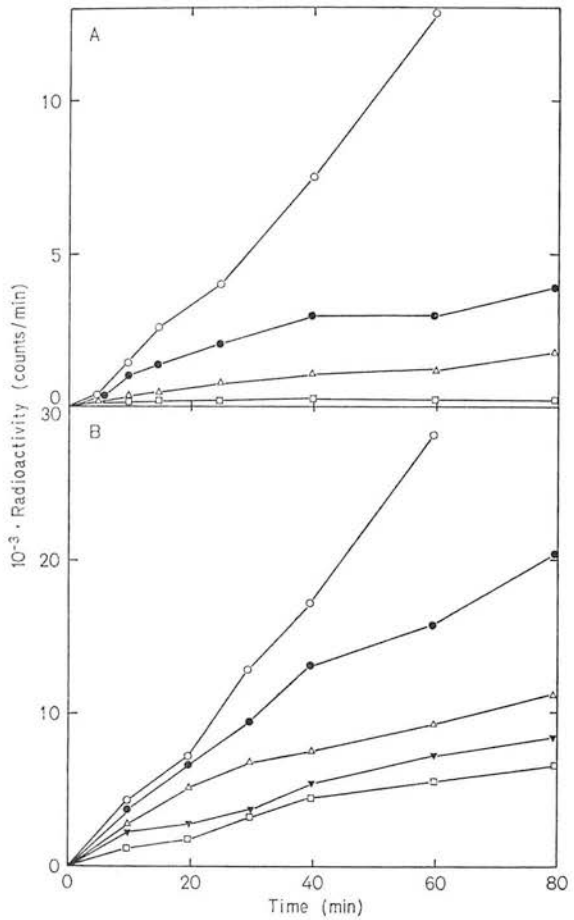

Fig. 1. *Effects of 8-hydroxyquinoline on (A) total RNA synthesis and (B) [³H]uridine uptake by yeast.* 8-Hydroxyquinoline was added to concentrations of 0 (○), 10 (●), 50 (△), 200 (▼) or 500 (□) μg/ml

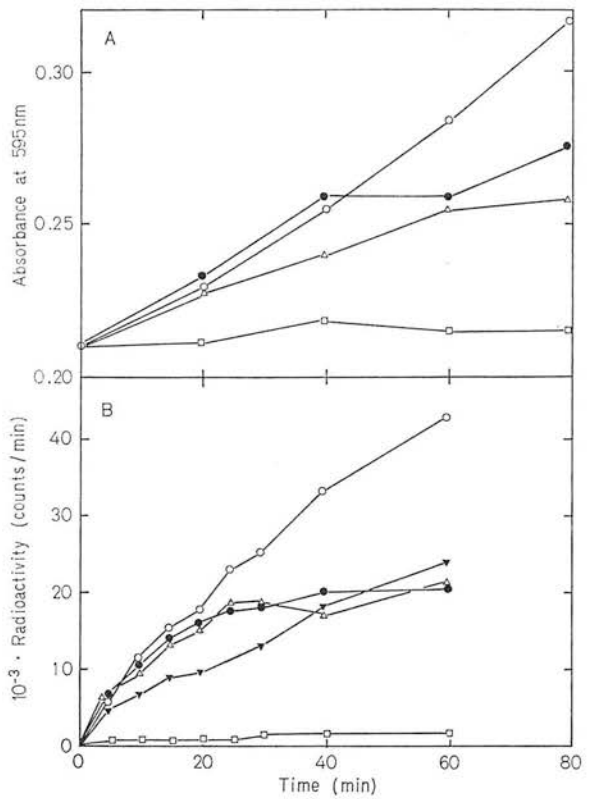

Fig. 2. *Effects of 8-hydroxyquinoline on (A) cell growth and (B) protein synthesis of yeast.* 8-Hydroxyquinoline was added to concentrations of 0 (○), 10 (●), 50 (△), 200 (▼) or 500 (□) μg/ml

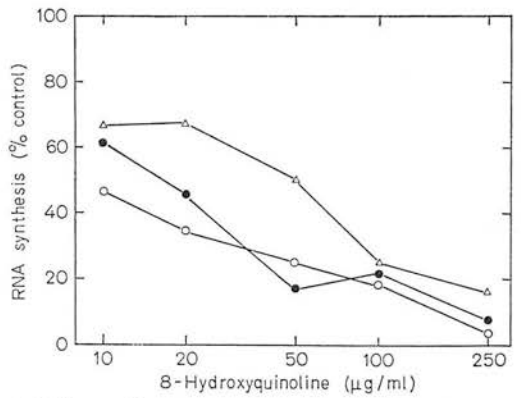

Fig. 3. *Effects of various concentrations of 8-hydroxyquinoline on yeast total RNA synthesis (○); synthesis of poly(A)-containing mRNA (●) and DNA synthesis (△).* Cells were labelled from 10–20 min after addition of 8-hydroxyquinoline, with [8-³H]adenine for DNA synthesis and with [5-³H]uridine for determinations of total RNA and poly(A)-mRNA synthesis. Results are expressed as percentages of incorporations in control cells with no 8-hydrodroxyquinoline

(Fig. 1 B). An estimate of the amount of ³H radioactivity present in the cold acid-soluble pool may be obtained by subtracting [³H]uridine incorporation (Fig. 1 A) from total [³H]uridine uptake (Fig. 1 B) [14]. It can be shown from the data of Fig. 1. That 8-hydroxyquinoline had comparatively much less effect on the entry of [³H]uridine into the cold-acid-soluble pool than on incorporation into RNA.

Cell growth was not immediately affected by low concentrations (10–50 μg/ml) of 8-hydroxyquinoline, but became inhibited after 20 to 40 min. Higher concentrations (500 μg/ml) caused an immediate cessation of growth (Fig. 2 A). At 10–50 μg/ml 8-hydroxyquinoline, protein synthesis was very little affected for 20 min, then quickly became almost completely inhibited (Fig. 2 B). 500 μg/ml caused immediate cessation of protein synthesis; at 200 μg/ml, the rate of protein synthesis was immediately reduced, but synthesis did continue for longer than at lower 8-hydroxyquinoline concentrations. DNA synthesis was inhibited by 8-hydroxyquinoline, but less than total RNA synthesis (Fig. 3).

These results show that low concentrations of 8-hydroxyquinoline selectively inhibit RNA synthesis.

In the first 20 min after addition to a concentration of 50 μg/ml, RNA synthesis was inhibited by 84%. The level of [^{3}H]uridine radioactivity in the cold acid-soluble pool (calculated from Fig. 1) was the same in control and 8-hydroxyquinoline-treated cells. Total uridine uptake was inhibited by 25%, cell growth by 10% and protein synthesis by 15%.

These results are consistent with a direct inhibition of RNA synthesis and exclude an indirect inhibition, for example by an effect on energy supply or isotope uptake. The eventual decline in protein synthesis, uridine uptake and cell growth in the presence of low concentrations of 8-hydroxyquinoline probably reflects a dependence of these aspects of metabolism on continued RNA synthesis.

The Mechanism of Inhibition of RNA Synthesis

Two lines of evidence suggest that the inhibition of RNA synthesis by 8-hydroxyquinoline is a result of chelation of divalent cations required for RNA synthesis. RNA polymerases are known to require divalent cations for activity [15—17].

Firstly, 8-hydroxyquinoline chelates because the —OH group is close to the ring —N atom of quinoline. Hydroxyquinolines with —OH groups more distant from the ring —N, such as 4-hydroxyquinoline, do not chelate. We tested 4-hydroxyquinoline on yeast, and found that even at 1000 μg/ml, it had no effect on RNA or protein synthesis.

Secondly, we argued that if the inhibition is by chelation, it should be possible to prevent inhibition by pre-saturating the chelating sites of 8-hydroxyquinoline with divalent cations before it enters the cell. Chelation by 8-hydroxyquinoline increases with increasing pH; the pK is 5.5 [18]. The pH of yeast culture medium, after growth of cells and the addition of 8-hydroxyquinoline, was 4.0—4.6. At these levels, most of the 8-hydroxyquinoline present would be unable to chelate divalent cations in the medium. No significant reduction in inhibition of RNA synthesis would be expected from raising the divalent cation concentration of the medium. Indeed the yeast culture medium already contained high divalent cation concentrations, including 125 mg/l Mg^{2+} and 4 mg/l Ca^{2+}. However, raising the pH of the medium should increase the ability of 8-hydroxyquinoline to chelate divalent cations in the medium and reduce the inhibition of RNA synthesis. This is confirmed by data shown in Fig. 4. 8-Hydroxyquinoline was added to cultures at 50 μg/ml, and the pH of the culture medium was adjusted with NaOH or CH_3COOH to 3.9, 4.6 or 5.7. The higher the pH of the medium, the lower was the inhibition of RNA synthesis. It was not possible to demonstrate a further reduction in the inhibition by 8-hydroxyquinoline at pH levels higher than 5.7, as control cells stopped making RNA above pH 6.0.

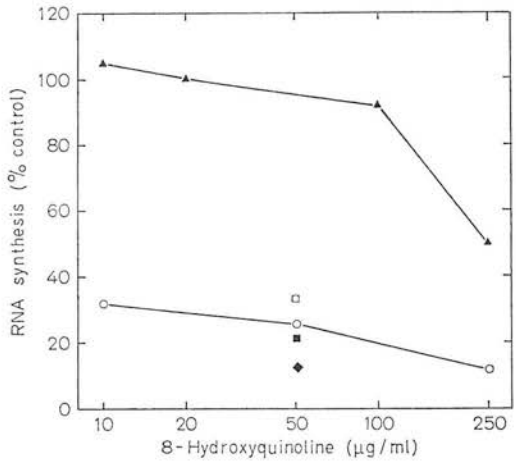

Fig. 4. *Effects of medium pH and effects of lowering the internal pH of cells, on inhibition of RNA synthesis in yeast by 8-hydroxyquinoline.* 8-Hydroxyquinoline was added to 50 μg/ml to yeast cultures grown at pH 5.3, and the pH of the medium was adjusted to 3.9 (◆), 4.6 (■) or 5.7 (□). Other cultures were exposed to various concentrations of 8-hydroxyquinoline after growth of cells in medium at pH 5.3 (○——○) or pH 4.0 (▲——▲). The pH 4.0 medium contained 10 times the normal acetate buffer concentration [9]. RNA was labelled with [5-^{3}H]uridine from 10—20 min after adding 8-hydroxyquinoline. Results are expressed as percentage of controls which were given the same growth and pH conditions as the 8-hydroxyquinoline treatments, but no 8-hydroxyquinoline

The mouse L-cells tested were grown in medium at pH 7.6. At this pH, 8-hydroxyquinoline chelates very strongly. The medium contained 88 mg/l Ca^{2+} and 20 mg/l Mg^{2+}. 8-Hydroxyquinoline inhibited RNA synthesis in L-cells when they were resuspended in medium lacking divalent cations (Fig. 5). There was no inhibition by 8-hydroxyquinoline when cells were resuspended in medium containing divalent cations.

These results show that inhibition of RNA synthesis by 8-hydroxyquinoline can be prevented by pre-saturating the chelating sites with divalent cations, and suggest that the mechanism of inhibition is by chelation of divalent cations within the cell. A later report will deal with the effects of 8-hydroxyquinoline on isolated RNA polymerases.

It follows that the internal pH of yeast must be sufficiently high to allow 8-hydroxyquinoline to chelate, and must also be significantly higher than the pH of the growth medium. The internal pH of budding yeast has been found by various methods to lie between 5.5 and 6.3 [19]. We have determined the internal pH of *S. pombe* by measuring the pH of washed, broken cells by the method of Conway and Downey [20] and

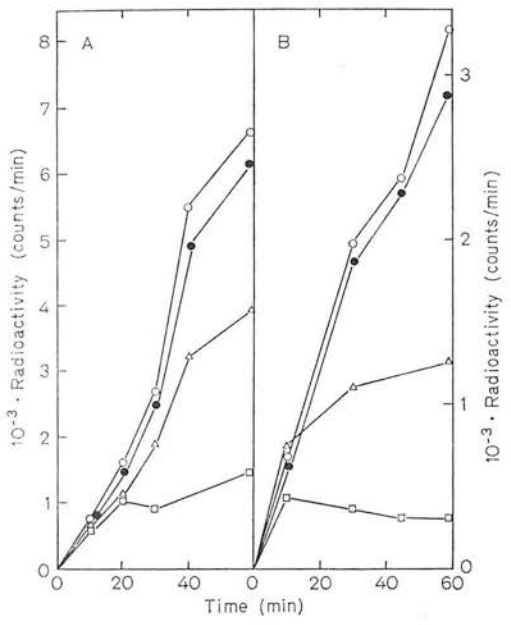

Fig. 5. *Effects of 8-hydroxyquinoline on (A) RNA and (B) protein synthesis by cultured mouse L-cells.* Cells were resuspended in medium containing divalent cations (●) or in medium lacking divalent cations (○, △, □). 8-Hydroxyquinoline was added to 0 (○), 50 (●, △) or 500 (□) μg/ml. Cells incubated without 8-hydroxyquinoline had the same rates of RNA and protein synthesis in medium with or without divalent cations

found values between 6.1 and 6.7. Thus pH conditions within the yeast cell and in the medium are such that 8-hydroxyquinoline is unlikely to chelate divalent cations in the medium, but is able to chelate within the cell.

As a further test, we argued that if the internal pH of the cell could be reduced, the inhibition of RNA synthesis by 8-hydroxyquinoline should also be reduced This was the case. When cells were grown in strongly buffered medium at pH 4.0, very high concentrations of 8-hydroxyquinoline were required to inhibit RNA synthesis (Fig. 4). The internal pH of cells grown at pH 4.0 was 0.5 pH units lower than that of control cells grown at pH 5.3. Gutstein [21] also found differences in the internal pH of budding yeast grown in media of differing pH.

The Selective Inhibition of RNA Synthesis

The pH dependence of chelation by 8-hydroxyquinoline suggests an explanation for the selective inhibition of RNA synthesis in yeast, and why another process requiring divalent cations, protein synthesis, was not inhibited. The experiments with L-cells (Fig. 5) show that there, protein synthesis was actually inhibited more than RNA synthesis. There is therefore nothing intrinsic about 8-hydroxyquinoline which makes it a selective inhibitor of RNA synthesis. But differences in local pH within the cell would cause differences in the ability of 8-hydroxyquinoline to chelate and hence lead to selective inhibition. We suggest that the pH in the nucleus of yeast, where RNA synthesis occurs, must be significantly higher than the pH in the cytoplasm, where protein synthesis occurs. Then low concentrations of 8-hydroxyquinoline would significantly reduce divalent cation concentration in the nucleus, but not in the cytoplasm. Unfortunately, it is at present technically difficult to obtain a meaningful picture of pH variation within the yeast cell. Conway and Downey [20] have shown that budding yeast has an "outer metabolic region" at a lower pH than the inner part of the cell.

It is also possible that the selective inhibition of RNA synthesis was a consequence of accumulation of 8-hydroxyquinoline in the nucleus at higher concentrations than in the cytoplasm.

Different Inhibition of High-Molecular-Weight RNA Synthesis

At high concentrations (50 μg/ml), 8-hydroxyquinoline inhibited the synthesis of all types of RNA (Fig. 6). However, at 10 μg/ml, there was a differential effect. The synthesis of rRNA was reduced by 50% during the labelling period, 10—20 min after addition of 8-hydroxyquinoline. The synthesis of polydisperse RNA, presumed to be mRNA, was also visibly reduced (Fig. 6). Examination of the synthesis of poly-(A)-containing mRNA [12] showed that it was inhibited by 8-hydroxyquinoline to a similar extent to rRNA synthesis (Fig. 3). In contrast, the synthesis of 5-S RNA and tRNA was not inhibited by low concentrations of 8-hydroxyquinoline (Fig. 6). This differential inhibition of higher-molecular-weight RNA synthesis is similar to our previous finding with the antibiotic lomofungin [7].

An explanation of this differential effect, consistent with what is known about eukaryote DNA-dependent RNA polymerases, is that yeast 5-S RNA and tRNA are made by a separate polymerase which has a different divalent cation requirement from the polymerases making rRNA and heterogeneous RNA. At least three separate nuclear RNA polymerases have been reported in yeast [16, 17, 23, 24]. Polymerases A and B appear similar to mammalian [23] or urchin [15] polymerases I and II, which make rRNA and heterogeneous RNA, respectively. The significance of yeast polymerase C has not yet been determined. All three yeast polymerases will operate with Mn^{2+}; only C is fully active with Mg^{2+} alone [17, 23]. 8-Hydroxyquinoline binds Mn^{2+} very much more strongly than it

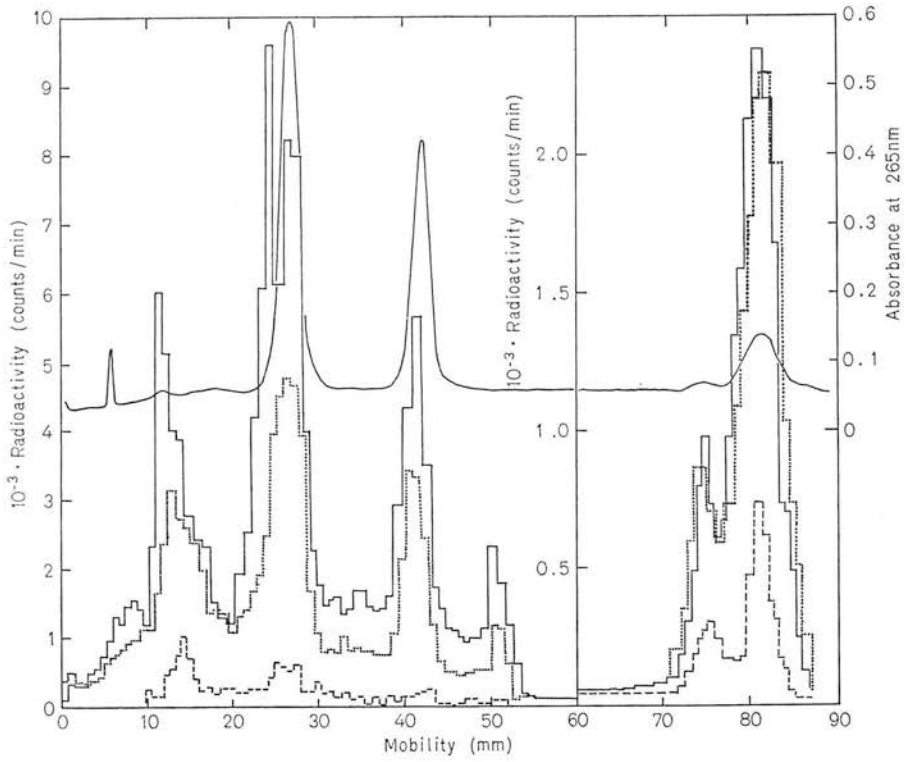

Fig. 6. *Polyacrylamide-gel electrophoresis of [5-^{3}H]uridine-labelled RNA from yeast.* The continuous line shows ultraviolet absorbance. The histograms show ^{3}H radioactivity. Cells were labelled with [5-^{3}H]uridine from 10—20 min after addition of 8-hydroxyquinoline to 0 (——), 10 (·····) or 50 (– – – –) μg/ml. The peaks are at 14 mm, r-RNA precursor; at 27 and 42 mm, 26 and 18-S rRNA; at 75 mm 5-S RNA and at 81 mm, tRNA. The accumulation of radioactivity at 50—54 mm marks the transition from 2.4% acrylamide (0—55 mm) to 10% acrylamide (55—100 mm) gel

binds Mg^{2+} [18]. Therefore at low 8-hydroxyquinoline concentrations, the consequence to the cell would be a drastic reduction of Mn^{2+} concentration and little change in Mg^{2+} concentration. This would permit activity of polymerase C but not of A and B. Our data (Fig. 6) are thus consistent with a separate polymerase in yeast synthesising 5-S RNA and tRNA; polymerase C is a likely candidate. Price and Penman [22] showed that in HeLa cells, 5-S RNA and tRNA are synthesised by a separate polymerase, probably polymerase III.

In connection with the possible involvement of separate polymerases in the synthesis of 5-S RNA and high-molecular-weight rRNAs, it is interesting that Rubin and Sulston [25] have found in budding yeast that the 5-S DNA cistrons are interspersed with, and closely linked to the cistrons coding for the precursor of 18 and 26-S rRNA.

8-Hydroxyquinoline and Protein Synthesis

The eventual inhibition of protein synthesis (Fig. 2B) noted at low 8-hydroxyquinoline concentrations was probably a result of existing mRNAs decaying and not being replaced because of the effects of 8-hydroxyquinoline on RNA synthesis. There was a fairly sudden change at 20—25 min after addition of the chelating agent, from almost no inhibition to virtually complete inhibition of leucine incorporation. This pattern is consistent with a pool of rapidly turning over messengers, in excess of the number actually being translated. Were such a situation to exist, no accurate estimate of messenger half-life could be made from leucine incorporation data, but the upper limit of messenger half-life can be placed at 20—25 min. This is in agreement with the time of 21 min suggested by Tønnesen and Friesen [5] for budding yeast.

CONCLUSION

8-Hydroxyquinoline at a concentration of about 50 μg/ml is a rapid and selective inhibitor of RNA synthesis in yeast. Of the other cellular processes measured, DNA synthesis was significantly inhibited, but protein synthesis, cell growth and isotope uptake were not. The selectivity of inhibition is most probably a result of a fortuitous difference in pH between nucleus

and cytoplasm. We are using 8-hydroxyquinoline in studies of mRNA turnover and to elucidate the relative importance of transcriptional and translational control of protein synthesis.

The effects of 8-hydroxyquinoline are remarkably similar to those of the antibiotic lomofungin [7,8]. This suggests a common mode of action. 8-Hydroxyquinoline has the advantages that it is readily available and its solubility is more than adequate. Lomofungin at the moment is not commercially produced and is an effective inhibitor only when used near saturating concentration.

This work was supported by the Medical Research Council and Science Research Council. We thank Prof. J. M. Mitchison for encouragement and advice and Miss A. M. Baker for technical assistance.

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Biochem. J. (1975) **147**, 401–410
Printed in Great Britain

The Mechanism of Inhibition of Ribonucleic Acid Synthesis by 8-Hydroxyquinoline and the Antibiotic Lomofungin

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(*Received* 14 *October* 1974)

RNA synthesis in yeast is rapidly inhibited by 8-hydroxyquinoline and the phenazine antibiotic lomofungin (5-formyl-1-methoxycarbonyl-4,6,8-trihydroxyphenazine). It is shown that lomofungin, like 8-hydroxyquinoline, is a chelating agent for bivalent cations. The mechanism of inhibition of RNA synthesis by lomofungin and 8-hydroxyquinoline was investigated in experiments with isolated *Escherichia coli* RNA polymerase. The results show that both inhibitors are capable of inhibiting polymerase activity solely by chelating the dissociable cations Mn^{2+} and Mg^{2+}. Evidence is presented which shows that inhibition may occur in the absence of any direct contact between the RNA polymerase or DNA template and the inhibitor. The possibility that inhibition might also occur by chelation of the Zn^{2+}, which is tightly bound to the polymerase, is discussed: it is concluded that lomofungin or 8-hydroxyquinoline is likely to inhibit the enzyme by removal of Mn^{2+} and Mg^{2+} before chelating the Zn^{2+}. On the basis of inhibition by chelation of Mn^{2+} and Mg^{2+}, explanations are proposed for why lomofungin and 8-hydroxyquinoline inhibit synthesis of ribosomal and polydisperse RNA more than that of 5S RNA and tRNA, and for why protein synthesis is not immediately inhibited in the intact yeast cell.

Lomofungin (5-formyl-1-methoxycarbonyl-4,6,8-trihydroxyphenazine) is an antibiotic from *Streptomyces lomodensis* (Johnson & Dietz, 1969). It rapidly inhibits RNA synthesis in yeast, but has no immediate effect on cell growth or protein synthesis (Gottlieb & Nicolas, 1969; Lampen *et al.*, 1973; Fraser *et al.*, 1973; Cannon *et al.*, 1973). Lomofungin is proving useful in studies of yeast RNA metabolism and enzyme synthesis, as other antibiotics such as actinomycin D fail to inhibit yeast RNA synthesis or do only at extremely high concentrations (Hartwell & McLaughlin, 1968; Soskova *et al.*, 1970; Medoff *et al.*, 1972; Mitchison *et al.*, 1973; Tønnesen & Friesen, 1973). An interesting feature of lomofungin action on yeast is that it strongly inhibits the synthesis of rRNA and polydisperse RNA, but has comparatively less effect on synthesis of 5S RNA and tRNA (Fraser *et al.*, 1973; Cano *et al.*, 1973; Cannon & Jimenez, 1974).

In the present paper we report experiments on the mechanism of inhibition of RNA synthesis by lomofungin. Cano *et al.* (1973) found that lomofungin inhibited the synthesis of RNA *in vitro* in systems using RNA polymerases extracted from *Saccharomyces cerevisiae* and *Escherichia coli*, and suggested that the RNA polymerase was the site of action of the antibiotic.

We found that 8-hydroxyquinoline has effects on RNA synthesis in yeast similar to those of lomofungin (Fraser & Creanor, 1974). This, together with certain similarities in the molecular structures of lomofungin and 8-hydroxyquinoline, suggested a common mode of action of the inhibitors. 8-Hydroxyquinoline is a chelating agent for bivalent cations (Hollingshead, 1954). This suggested that lomofungin and 8-hydroxyquinoline might inhibit RNA synthesis by chelating bivalent cations required for RNA polymerase activity.

Two distinct types of bivalent cations are known to be involved in RNA polymerase activity: the dissociable cations Mn^{2+} and Mg^{2+} (Roeder & Rutter, 1969; Burgess, 1971; Ponta *et al.*, 1971), and Zn^{2+}, which is tightly bound to the enzyme (Scrutton *et al.*, 1971; Valenzuela *et al.*, 1973). Pavletich *et al.* (1974) have suggested that lomofungin acts by chelating the Zn^{2+}. In the present paper we show that lomofungin can chelate Mn^{2+} and Mg^{2+}. We report that lomofungin and 8-hydroxyquinoline are capable of inhibiting RNA polymerase solely by chelating the Mn^{2+} and Mg^{2+}, and that inhibition of enzyme activity can occur in the absence of direct contact between inhibitor and enzyme. The question of whether inhibition of RNA polymerase activity *in vivo* is a consequence of chelation of dissociable bivalent cations or Zn^{2+} is discussed. The knowledge that 8-hydroxyquinoline and lomofungin inhibit by chelation permits interpretation of some other aspects of their effects on yeast.

Materials and Methods

Lomofungin

Lomofungin was a gift from Dr. G. B. Whitfield of the Upjohn Co., Kalamazoo, Mich., U.S.A. It was purified from a contaminant, probably a bivalent or tervalent cation: the lomofungin was dissolved at 300 μg/ml in 10mM-Tris adjusted to pH7.95 at 20°C with 1M-HCl. Insoluble material was removed by centrifugation for 1min at 12000g ($r_{av.}$ 4.5cm). 8-Hydroxyquinoline was added to 5mM, then (with any chelates formed) removed by chloroform extraction. The pH of the aqueous phase was lowered to 3.5 by addition of acetic acid. Most of the lomofungin precipitated was collected by centrifugation as above and dried under vacuum.

RNA polymerase assay

RNA polymerase (RNA nucleotidyltransferase, EC 2.7.7.6) from *E. coli* strain M.R.E. 600 was purchased from Boehringer Corp. (London), London W.5, U.K.

RNA polymerase activity was measured by the incorporation of [^{3}H]CTP radioactivity into trichloroacetic acid-insoluble material. The standard incubation mixture contained, in a total volume of 0.5ml, 50mM-Tris–HCl buffer, pH7.95 at room temperature (20°C); 1.5mM-$MnCl_2$; 1.0mM-$MgCl_2$; 1.0mM-2-mercaptoethanol; 50mM-KCl; 0.1mM each of ATP, GTP and UTP; 0.025mM- [^{3}H]CTP (5 μCi) (The Radiochemical Centre, Amersham, Bucks., U.K.); 42 μg of denatured calf thymus DNA as template. The reaction was started by addition of 5 μg of RNA polymerase. After various times of incubation at 37°C, 20 μl samples were withdrawn and mixed with 40 μg of yeast non-radioactive carrier RNA and 0.5ml of ice-cold 10% (w/v) trichloroacetic acid containing 40mM-sodium pyrophosphate. The precipitate was collected by filtration on Whatman GF/A glass-fibre paper, washed three times with ice-cold 10% trichloroacetic acid–40mM-sodium pyrophosphate and twice with 80% (v/v) ethanol containing 0.1M-NaCl. The filters were dried and incorporation of radioactivity into acid-insoluble polynucleotides was determined by counting in 5ml of 0.5% butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole]–toluene scintillator in a Packard liquid-scintillation counter. Counting efficiency was approx. 10%. At least 1000 counts above background were collected for each sample.

Single-point assays of enzyme activity were carried out by incubating 100 μl reaction mixtures for 5min. The final concentrations of all the constituents were as described above. Carrier RNA (40 μg) and 0.5ml of trichloroacetic acid were added to the entire reaction mixture to stop the reaction, and total incorporation was determined as above.

DNA-independent incorporation was measured in assays lacking DNA.

Purified lomofungin was dissolved directly in the polymerase reaction mixture before addition of polymerase, to give the required final lomofungin concentration.

8-Hydroxyquinoline was dissolved in ethanol at 100 times the required final concentration, and 0.01 vol. was added to the reaction mixture before addition of polymerase. Control experiments showed that 1% ethanol had no effect on polymerase activity.

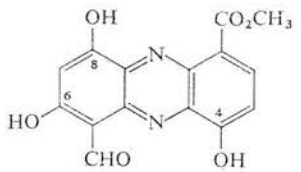
U.v.-absorption spectra

U.v-absorption spectra of lomofungin and 8-hydroxyquinoline were measured in a Unicam SP. 800 recording spectrophotometer, in 10mm path-length cells.

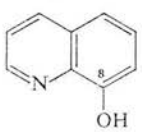
Results

Evidence that lomofungin is a chelating agent

The structural formulae of lomofungin (I) and 8-hydroxyquinoline (II) are shown. 8-Hydroxyquinoline is able to chelate bivalent cations because of the location of the hydroxyl group relative to the ring nitrogen. The hydroxyl group acts as an acid; as the pH rises it dissociates to $-O^-$. Bivalent cations are bound by the dissociated acid group and the lone pair of electrons borne by the nitrogen atom (Hollingshead, 1954).



(I) Lomofungin



(II) 8-Hydroxyquinoline

The structural formula of lomofungin shows two possible chelating sites, namely where the hydroxyl groups at positions 4 and 8 are located relative to ring nitrogen atoms (as in 8-hydroxyquinoline). Four lines of evidence confirm that lomofungin can chelate bivalent cations.

(1) 8-Hydroxyquinoline chelates of certain metal cations, such as Mn^{2+}, are insoluble within certain pH ranges (Hollingshead, 1954). Addition of Mn^{2+} (1.5mM) to lomofungin solution (1.25mM in 10mM-Tris–HCl buffer at pH7.95) caused formation of a heavy precipitate.

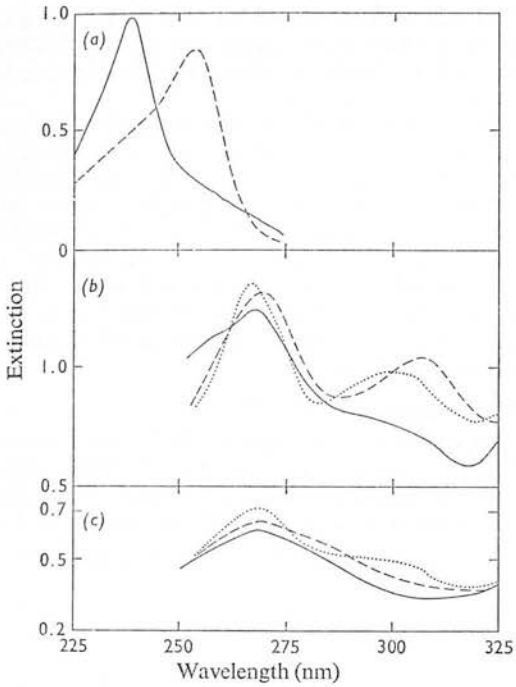


Fig. 1. *U.v.-absorption spectra of 8-hydroxyquinoline and lomofungin*

(*a*) 0.035mM-8-Hydroxyquinoline in 10mM-Tris–HCl buffer, pH7.95, alone (——) and with 5mM-$MgCl_2$ (– – – –). (*b*) 0.025mM Purified lomofungin in 10mM-Tris–HCl buffer, pH7.95, alone (——) and plus 0.05mM-$MnCl_2$ (– – – –) or 0.1mM-$MgCl_2$ (· · · ·). (*c*) 0.012mM Purified (——) and commercial (– – – –) lomofungin in 20mM-sodium acetate adjusted to pH5.5 with 1M-acetic acid, and purified lomofungin plus 0.05mM-$MgCl_2$ (· · · ·).

(2) Chelation of bivalent cations by 8-hydroxyquinoline results in a change in the u.v.-absorption spectrum (Fig. 1*a*). Fig. 1(*b*) shows the u.v.-absorption spectrum of purified lomofungin. Addition of Mn^{2+} or Mg^{2+} caused changes in the absorption spectrum, with increased extinction in the 265–275 and 295–315nm regions. Univalent cations (Na^+, K^+) did not cause a change in the absorption spectrum. The absorption spectrum of the lomofungin-plus-bivalent-cation form could be restored to the free-lomofungin form by addition of EDTA to a concentration exceeding that of the bivalent cation.

(3) Fig. 2 shows the pH-dependence of the change in extinction of lomofungin at 308nm caused by extinction of lomofungin at 308 nm caused by addition of Mn^{2+}. Taking the change in extinction at 308nm as a measure of the amount of chelation, the results indicate that chelation commenced at about pH4.5, and that chelation capacity increased up to about pH7. These results are consistent with a dependence of chelation on dissociation of acidic hydroxyl groups, and suggest that half of the hydroxyl groups are dissociated and available for chelation at pH5.6. The pK_a for the dissociation of the hydroxyl group of 8-hydroxyquinoline is 5.5 (Hollingshead, 1954; O'Sullivan, 1969).

(4) We attempted to measure the stoicheiometry of chelation of bivalent cations by lomofungin, by measuring the change in u.v. absorption when increasing amounts of Mn^{2+} or Mg^{2+} were added to lomofungin solutions. The measurements were made at pH7.95, where almost all the hydroxyl groups should be dissociated. Fig. 3 shows that with 0.025mM-lomofungin, a maximum increase in the extinction at 308nm was obtained with 0.05mM-Mn^{2+}. These data suggest that at saturation, two Mn^{2+} cations are bound per molecule of lomofungin, a result consistent with the two binding sites suggested by the structural formula. The slight decline in the extent of the change in extinction at 308nm at higher concentrations of Mn^{2+} was caused by some precipitation of the lomofungin–Mn^{2+} chelate.

A rather higher concentration of Mg^{2+} (0.1mM) was required to produce the maximum change of extinction of 0.025mM-lomofungin (Fig. 3). This result could mean that lomofungin can bind more than two Mg^{2+} cations per molecule, but is more likely to mean that the stability constant of the lomofungin–Mg^{2+} chelate is comparatively low, and that higher Mg^{2+} concentrations are required to ensure complete occupation of the available chelating sites. It is

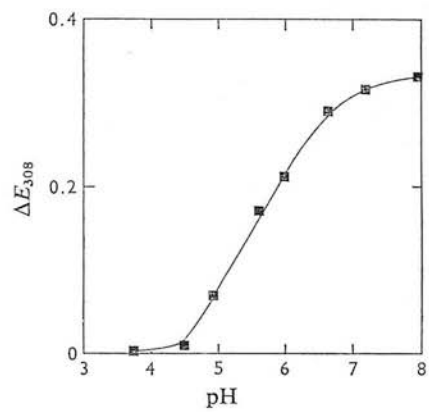


Fig. 2. *pH-dependence of chelation of Mn^{2+} by lomofungin*

Changes in the extinction at 308nm caused by adding 0.05mM-$MnCl_2$ to 0.025mM-purified lomofungin, at various pH values. Solutions were buffered by 20mM-sodium acetate adjusted with 1M-acetic acid to pH values between 3.7 and 6.0, by 20mM-2-(*N*-morpholino) ethanesulphonic acid adjusted with 1M-NaOH to pH6.6 or 7.2, or by 20mM-Tris–HCl buffer, pH7.95.

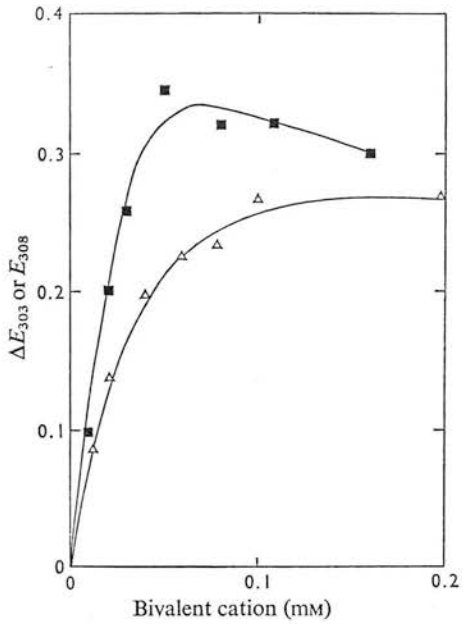


Fig. 3. *Stoicheiometry of chelation of* Mn^{2+} (■) *and* Mg^{2+} (△) *by lomofungin*

Various concentrations of $MnCl_2$ or $MgCl_2$ were added to 0.025mM-lomofungin in 10mM-Tris–HCl buffer, pH7.95. Chelation was measured by the change in extinction at 308nm (Mn^{2+}) or 303nm (Mg^{2+}).

consistent with this explanation that 8-hydroxyquinoline also binds Mn^{2+} more strongly than Mg^{2+} (O'Sullivan, 1969).

Purification of lomofungin

The experiments in this paper were carried out with lomofungin purified from the commercial sample. The absorption spectrum of commercial lomofungin (Fig. 1*c*) was similar to that obtained when bivalent cation was added to purified lomofungin. The absorption spectra shown in Fig. 1(*c*) were measured at pH5.5, as the contaminant–lomofungin complex is more soluble at pH5.5 than at pH7.95.

The absorption spectrum of commercial lomofungin could be shifted to the purified lomofungin form by addition of EDTA. These data therefore suggest that the commercial lomofungin contained a bivalent (or tervalent) cation as impurity. This impurity was found to inhibit RNA polymerase activity under certain circumstances, for example in the presence of very high concentrations of Mn^{2+} and Mg^{2+} (Fig. 7), which presumably liberated the impurity from lomofungin by competition for the chelating sites. At the normal bivalent cation concentration of the standard polymerase reaction mixture, the impurity remained bound to the lomofungin, and RNA polymerase activity was affected similarly by commercial and purified lomofungin, as shown in Fig. 6.

The purification scheme for lomofungin described in the Materials and Methods section attempted to remove the contaminant in three ways: by dissolving the lomofungin at high pH and high concentration, and removing insoluble chelates; by competing for cations with relatively high concentrations of 8-hydroxyquinoline, and by decreasing the pH to 3.5, where lomofungin does not chelate (Fig. 2), and where the antibiotic is insoluble, leaving the liberated cations in solution.

RNA polymerase assay

Fig. 4 shows a time-course of the incorporation of [^{3}H]CTP radioactivity into acid-insoluble polynucleotide by *E. coli* RNA polymerase. Depending on the batch of enzyme used, incorporation was

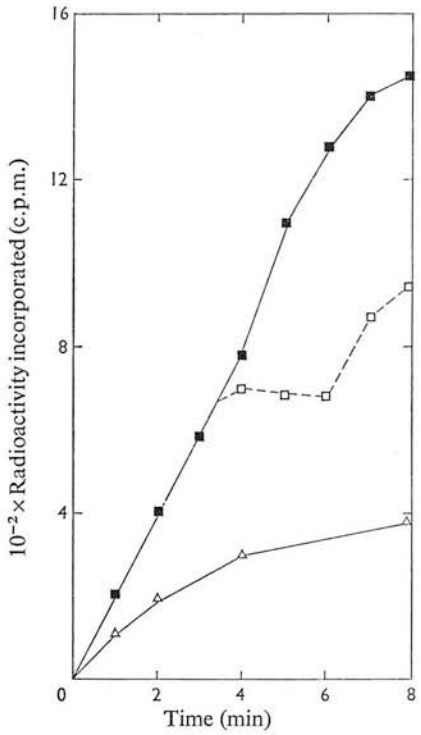


Fig. 4. *Kinetics of incorporation of* [^{3}*H*]*CTP by isolated E. coli RNA polymerase*

△, DNA-independent incorporation; ■, DNA-dependent incorporation, found by subtracting DNA-independent incorporation from total incorporation; □, DNA-dependent incorporation in an incubation to which 1.0mM-lomofungin was added after 3.5min and an additional 1.5mM-$MnCl_2$ and 1.0mM-$MgCl_2$ were added after 6min.

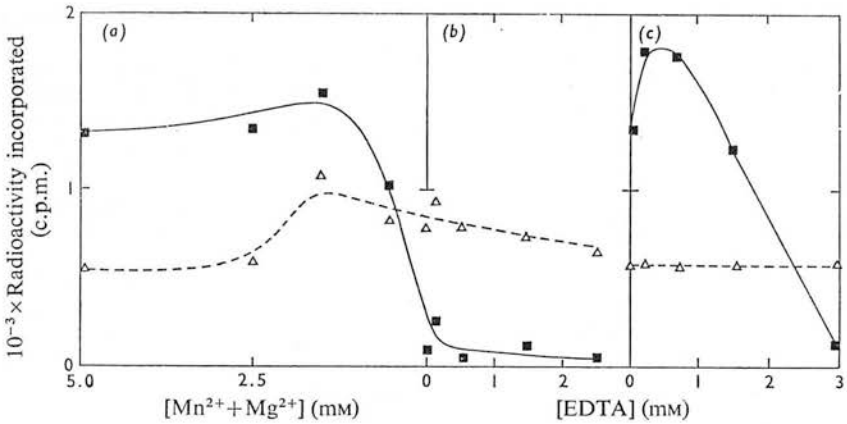


Fig. 5. *Bivalent-cation requirements of E. coli RNA polymerase*

■, DNA-dependent incorporation; △, DNA-independent incorporation in a 5min incubation. (*a*) Incubations containing no EDTA and various concentrations of bivalent cation in the molar ratio 1.5 $MnCl_2$:1.0 $MgCl_2$. (*b*) Incubations containing no bivalent cation and various concentrations of EDTA. (*c*) Incubations containing 1.5mM-$MnCl_2$, 1.0mM-$MgCl_2$ and various concentrations of EDTA.

linear with time for 7 to 40min. Also depending on the batch of enzyme, between 10 and 30% of total incorporation was found to be independent of a DNA template. Negligible amounts of radioactivity were rendered acid-insoluble when no enzyme was present.

Fig. 5 shows the bivalent-cation requirements for enzyme activity. DNA-independent incorporation was relatively independent of bivalent cation concentration (Fig. 5*a*) and also continued in the complete absence of bivalent cations (Fig. 5*b*). The cause of this DNA-independent incorporation is not fully understood.

To test whether lomofungin was capable of inhibiting RNA polymerase activity solely by removing bivalent cations, it was necessary to correct for this amount of bivalent cation-independent DNA-independent incorporation. This was done by running each assay in parallel, with and without DNA. The amount of DNA-dependent incorporation was found by subtracting DNA-independent incorporation from total incorporation.

DNA-dependent incorporation did not occur in the absence of bivalent cations (Fig. 5*b*); it rose to a maximum at a total $Mn^{2+}+Mg^{2+}$ concentration of 1.5mM (Fig. 5*a*), then remained fairly steady with increasing bivalent cation concentration over the range tested. In the presence of 1.5mM-Mn^{2+} and 1.0mM-Mg^{2+}, DNA-dependent RNA polymerase activity could be decreased by addition of EDTA. At low concentrations of added EDTA, up to 0.5mM, enzyme activity was increased with some batches of enzyme (Fig. 5*c*). This was presumably by removal of traces of toxic metal-cation contaminants in the assay mixture.

Effects of lomofungin and 8-hydroxyquinoline on RNA polymerase activity

As agents chelating Mn^{2+} and Mg^{2+}, lomofungin and 8-hydroxyquinoline would be expected to inhibit DNA-dependent RNA polymerase by decreasing the concentrations of these cations to values insufficient for enzyme activity (Fig. 5*a*). However, this does not exclude the possibility that lomofungin and 8-hydroxyquinoline might inhibit the enzyme activity in another way, possibly by binding to the DNA, by direct interaction with the polymerase as suggested by Cano *et al.* (1973), or by chelating the Zn^{2+} tightly bound to the polymerase (Pavletich *et al.*, 1974). If lomofungin and 8-hydroxyquinoline are capable of inhibiting RNA polymerase by chelation of the dissociable bivalent cations, three predictions should be fulfilled. (1) The concentration of inhibitor giving complete inhibition of polymerase activity should be that required to lower the $Mn^{2+}+Mg^{2+}$ concentration to a value insufficient for enzyme activity. (2) The inhibition of polymerization should be reversed by addition of more Mn^{2+} and Mg^{2+}. (3) Inhibition should occur in the absence of direct contact between inhibitor and DNA or polymerase. These predictions have been tested experimentally.

(1) The effects of various concentrations of lomofungin or 8-hydroxyquinoline on DNA-dependent DNA polymerase activity are shown in Fig. 6. The two dose–response curves share certain features. At low concentrations of lomofungin or 8-hydroxyquinoline, there was no inhibition of enzyme activity; indeed, with some samples of enzyme, a small stimulation was found. Inhibition commenced at concentrations of 0.2mM-lomofungin and 1.0mM-

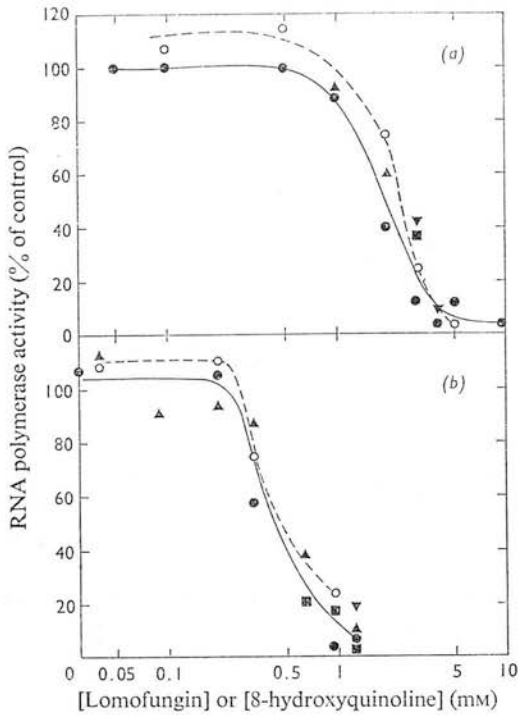


Fig. 6. *Effects of various concentrations of* (*a*) *8-hydroxyquinoline and* (*b*) *lomofungin on DNA-dependent RNA polymerase activity*

Continuous lines and solid symbols show results obtained when 8-hydroxyquinoline or lomofungin was present during the enzyme assay. The different solid symbols represent results from different batches of enzyme. For lomofungin, results shown by (▲) were obtained with commercial lomofungin; those shown by (●, ■, ▼) were obtained with purified lomofungin. The broken lines and open circles show results obtained when 8-hydroxyquinoline or lomofungin was removed from the incubation before addition of DNA and RNA polymerase, as explained in the Results section. All data are expressed as percentages of activity in control cultures containing no inhibitor. The incubation time was 5 min.

8-hydroxyquinoline, and was complete at about 1.25 mM-lomofungin and between 3 and 5 mM-8-hydroxyquinoline.

The total bivalent cation concentration of the reaction mixture was 2.5 mM (1.5 mM-Mn^{2+} + 1.0 mM-Mg^{2+}). The concentration of 8-hydroxyquinoline required for complete chelation of the Mn^{2+} and Mg^{2+} is 5 mM, since each bivalent cation can bind two molecules of 8-hydroxyquinoline in the chelate (Hollingshead, 1954). The data of Fig. 3 suggest that 1.25 mM-lomofungin should chelate most of the 2.5 mM bivalent cation, as each lomofungin molecule binds two Mn^{2+} ions or rather less than two Mg^{2+} ions at pH 7.95.

As both lomofungin (Fig. 3) and 8-hydroxyquinoline (O'Sullivan, 1969) bind Mn^{2+} more strongly than Mg^{2+}, it is likely that Mn^{2+} would be removed more effectively than Mg^{2+} when the total chelator concentration is less than required for complete chelation of Mn^{2+} and Mg^{2+}. Mg^{2+} alone is much less effective as an activator of RNA polymerase than Mn^{2+} + Mg^{2+}. Pavletich *et al.* (1974) reported maximum RNA polymerase activity with 15–20 mM-Mg^{2+}, whereas maximum activity was obtainable with 1.5–2.5 mM of the combined cations (Fig. 5*a*). The concentrations of lomofungin and 8-hydroxyquinoline required to give near-complete inhibition of RNA polymerase activity (Fig. 6) were thus slightly less than those necessary for complete removal of all Mn^{2+} and Mg^{2+}. However, these inhibitor concentrations should have removed practically all of the Mn^{2+} and much of the Mg^{2+}, leaving the polymerase with too little Mg^{2+} for significant activity.

(2) Inhibition of RNA polymerase activity by 8-hydroxyquinoline could be prevented by adding extra Mn^{2+} + Mg^{2+} to the incubations (Fig. 7*a*). At the highest inhibitor concentration, it was not possible to get complete reversal of inhibition.

It proved less easy to demonstrate reversibility of inhibition by lomofungin. With the unpurified lomofungin, addition of extra Mg^{2+} and Mn^{2+} typically increased the inhibition of RNA polymerase activity (Fig. 7*b*), probably by releasing the presumed toxic contaminant from lomofungin (Fig. 1*c*). Very high concentrations of extra Mn^{2+} and Mg^{2+} gave some reversal of inhibition (Fig. 7*b*). The inhibition of RNA polymerase by purified lomofungin was readily reversible by addition of extra Mn^{2+} and Mg^{2+}.

The experiments described in Figs. 6 and 7 were carried out by adding polymerase to incubation mixtures already containing lomofungin or 8-hydroxyquinoline and bivalent cations. Fig. 4 shows that when purified lomofungin was added to an incubation in which the polymerase was already in action, inhibition occurred immediately. Polymerase activity recommenced immediately on addition of extra Mn^{2+} + Mg^{2+} to the incubation.

(3) As a check on the ability of 8-hydroxyquinoline and lomofungin to inhibit the polymerase solely by chelation of Mn^{2+} and Mg^{2+}, polymerase activity was assayed in reaction mixtures from which lomofungin or 8-hydroxyquinoline had been removed before addition of DNA and enzyme.

Incomplete polymerase-assay mixtures were prepared, containing the normal final amounts of Tris buffer, KCl, mercaptoethanol and bivalent cations, but lacking DNA, nucleoside triphosphates and RNA polymerase. 8-Hydroxyquinoline or lomofungin

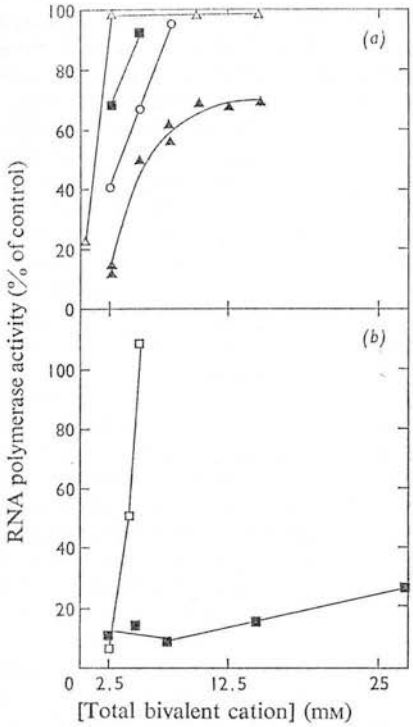


Fig. 7. *Reversal of inhibition of RNA polymerase by 8-hydroxyquinoline or lomofungin*

Bivalent cations in the molar ratio 1.5 Mn^{2+}: 1.0 Mg^{2+} were added to various final total concentrations before addition of RNA polymerase to reaction mixtures containing lomofungin or 8-hydroxyquinoline. (*a*) △, DNA-dependent RNA polymerase activity in control incubations containing no inhibitor, and polymerase activity in the presence of (■) 2mM-, (○) 3mM- and (▲) 4mM-8-hydroxyquinoline. (*b*) RNA polymerase activity in incubations containing 1.0mM purified (□) or commercial (■) lomofungin. All data are expressed as percentages of incorporation in a control assay with 2.5mM total bivalent cation. The incubation time was 5min.

was added to various concentrations. 8-Hydroxyquinoline and its chelates were removed by shaking the incomplete reaction mixtures twice with equal volumes of chloroform. The residual chloroform was removed under vacuum. Examination of the u.v.-absorption spectrum of the incomplete reaction mixture after 8-hydroxyquinoline treatment and chloroform extraction confirmed that removal of 8-hydroxyquinoline had been complete. Lomofungin was more difficult to extract, as it does not readily partition into organic solvents at high pH. Insoluble chelates formed in incomplete reaction mixtures with high lomofungin concentrations at pH7.95 were removed by centrifugation. The pH was then lowered to 5.5 by addition of acetic acid; most of the remaining lomofungin precipitated and was removed by centrifugation. The reaction mixture was then shaken with chloroform, which removed the last of the lomofungin, and the pH was restored to 7.95 with Tris. Incomplete reaction mixtures, without added chelating agent, were extracted by both above schemes as controls.

RNA, nucleoside triphosphates and RNA polymerase were added to the chelating-agent-treated or control partial incubation mixtures, and polymerase activity was measured as usual. The results were dose–response curves similar to those obtained when lomofungin or 8-hydroxyquinoline was present during the polymerase assay (Fig. 6). These results therefore prove that lomofungin and 8-hydroxyquinoline are capable of inhibiting RNA polymerase solely by chelation of Mn^{2+} and Mg^{2+}, and show that inhibition can occur in the absence of direct contact between the chelating agent and the DNA or RNA polymerase.

Discussion

We have shown that lomofungin can chelate Mg^{2+} and Mn^{2+} (Fig. 3). 8-Hydroxyquinoline will also chelate these cations (O'Sullivan, 1969). The *E. coli* RNA polymerase used in our experiments was inactive in the absence of Mn^{2+}+Mg^{2+} (Fig. 5*a*). The data for polymerase activity with different concentrations of lomofungin or 8-hydroxyquinoline (Fig. 6) show that complete inhibition was achieved at the inhibitor concentrations required for chelation of most of the Mn^{2+} and Mg^{2+} present. Fig. 6 also shows that the same dose–response curves were obtained when there was no direct contact between enzyme and inhibitor. Finally, enzyme activity could be restored by addition of extra Mn^{2+} and Mg^{2+} to reactions inhibited by lomofungin or 8-hydroxyquinoline (Figs. 4 and 7). These results show unequivocally that lomofungin and 8-hydroxyquinoline are capable of inhibiting isolated *E. coli* RNA polymerase solely by chelating essential Mn^{2+} and Mg^{2+}. Our data do not exclude the possibility that other mechanisms of inhibition by lomofungin and 8-hydroxyquinoline might exist *in vivo*.

In contrast with our conclusions, Pavletich *et al.* (1974) claim to have shown that lomofungin does not act by chelating Mg^{2+} or Mn^{2+}. They suggested that lomofungin inhibits RNA synthesis by preventing the initiation of transcription, by chelating the Zn^{2+} which is tightly bound to the RNA polymerase (Scrutton *et al.*, 1971). We now discuss experimental evidence against these conclusions.

The suggestion by Pavletich *et al.* (1974) that lomofungin does not act by chelating Mn^{2+} or Mg^{2+} stems from their finding that yeast cells could be protected from inhibition of growth by lomofungin, by preincubation for 2h in culture medium con-

taining extra Cu^{2+}, Zn^{2+} or Fe^{2+}, but no protection was obtained when the medium contained extra Mn^{2+} or Mg^{2+}. In contrast, we have found that the inhibition of RNA synthesis in yeast by lomofungin does bear an inverse relationship to the Mg^{2+} concentration in the medium, but growth periods of longer than 2h under different Mg^{2+} concentrations are required to show the maximum effect (R. S. S. Fraser & J. Creanor, unpublished work). The protection of yeast from inhibition afforded by Cu^{2+}, Zn^{2+} and Fe^{2+} occurs because lomofungin chelates these cations (Pavletich *et al.*, 1974). In the cell this must result in a decrease in the concentration of free lomofungin available to inhibit RNA synthesis.

Pavletich *et al.* (1974) investigated the effects of Mg^{2+} concentration on the inhibition of isolated *E. coli* RNA polymerase by lomofungin, and found a significant increase in the activity of lomofungin-inhibited enzyme with higher Mg^{2+} concentrations. We attribute their failure to obtain complete recovery of polymerase activity to the use of unpurified lomofungin, from which high Mg^{2+} concentrations probably cause the release of a metal cation toxic to polymerases (Fig. 1*c*). Fig. 7 shows that with purified lomofungin, addition of extra Mn^{2+} and Mg^{2+} gave much higher recovery of polymerase activity than when unpurified lomofungin was used.

Pavletich *et al.* (1974) quoted no direct experimental evidence that lomofungin does not chelate Mn^{2+} and Mg^{2+}; we have shown that lomofungin does chelate these cations (Figs. 1 and 3). We therefore consider that these results of Pavletich *et al.* (1974) do not exclude the chelation of Mn^{2+} and Mg^{2+} by lomofungin as a means of inhibition of RNA polymerase.

The activity of RNA polymerase depends on the dissociable cations Mn^{2+} and Mg^{2+}, and on Zn^{2+}, which is tightly bound to the enzyme (Scrutton *et al.*, 1971; Valenzuela *et al.*, 1973). The ability of lomofungin and 8-hydroxyquinoline to chelate all three bivalent cations poses the question of whether the inhibition of RNA polymerase by these agents is a result of chelation of Zn^{2+}, the dissociable cations, or all three. Our data show that inhibition by chelation of Mn^{2+} and Mg^{2+} alone is possible, but do not rule out the chelation of Zn^{2+} as a further contribution to inhibition. Pavletich *et al.* (1974) concluded that inhibition was by chelation of Zn^{2+}, from experiments showing that Zn^{2+} and Cu^{2+} prevented inhibition of RNA synthesis in yeast by lomofungin, and from a demonstration that RNA polymerase pretreated with lomofungin failed to form the DNA–RNA polymerase initiation complex. Three types of experimental evidence suggest that chelation of Zn^{2+} is unlikely to contribute to the primary mechanism of inhibition of RNA polymerase by lomofungin or 8-hydroxyquinoline.

(1) Inhibition of RNA polymerase by specific chelation of Zn^{2+} is very slow, with reported delays before full inhibition of 5–10min for *E. coli* RNA polymerase (Scrutton *et al.*, 1971) and 2h for eukaryote RNA polymerases (Valenzuela *et al.*, 1973). Inhibition by chelation of Zn^{2+} may be slow both because Zn^{2+} is involved in chain initiation rather than elongation, and because the Zn^{2+} is very tightly bound to the enzyme (Scrutton *et al.*, 1971) In contrast, lomofungin inhibited isolated *E. coli* RNA polymerase immediately (Fig. 4). Cano *et al.* (1973) also reported an immediate inhibition of isolated yeast RNA polymerase by lomofungin, and suggested that lomofungin inhibited chain elongation.

(2) The chelating agent of choice in studies of polymerase-bound Zn^{2+} is *o*-phenanthroline, which chelates Zn^{2+} but not Mg^{2+} (O'Sullivan, 1969). Hence *o*-phenanthroline does not interfere with the dissociable bivalent-cation requirement of the polymerase. 8-Hydroxyquinoline and EDTA chelate Zn^{2+} more strongly than *o*-phenanthroline does, but 8-hydroxyquinoline and EDTA also chelate Mg^{2+}. Scrutton *et al.* (1971) and Valenzuela *et al.* (1973) found that RNA polymerase was strongly inhibited by 0.5–1.0mM-*o*-phenanthroline, in the presence of 5–10mM-Mg^{2+}. Scrutton *et al.* (1971) found that 1mM-EDTA or -8-hydroxyquinoline did not inhibit polymerase activity. Valenzuela *et al.* (1973) reported that up to 10mM-EDTA or 8-hydroxyquinoline was required for strong inhibition. The clear implication of these results is that Mg^{2+} saturates the chelating sites of EDTA or 8-hydroxyquinoline, and prevents chelation of the Zn^{2+}. The concentrations of EDTA or 8-hydroxyquinoline reported by Valenzuela *et al.* (1973) to give strong inhibition of RNA polymerase activity were those required for chelation of the Mg^{2+}.

(3) Pavletich *et al.* (1974) found that incubation of RNA polymerase with lomofungin in the absence of Mg^{2+} prevented formation of the initiation complex between DNA and the polymerase. This result shows only that lomofungin is capable of chelating the Zn^{2+} of the polymerase in the absence of competing bivalent cations. It does not show that lomofungin will prevent formation of the initiation complex *in vivo* or in a complete system containing Mg^{2+} *in vitro*. Indeed, Pavletich *et al.* (1974) found that when lomofungin was preincubated with DNA, with subsequent addition of Mg^{2+} and polymerase, there was no inhibition of formation of the initiation complex by lomofungin. This result strongly suggests that Mg^{2+} prevented the chelation of polymerase-bound Zn^{2+} by lomofungin.

From these considerations, and from the experimental evidence reported in this paper, we suggest that the primary mechanism of inhibition of RNA polymerase by lomofungin and 8-hydroxyquinoline is by the chelation of the dissociable bivalent cations Mn^{2+} and Mg^{2+}, and not by chelation of the Zn^{2+} tightly bound to the enzyme. Our data do not exclude

the possibility that concentrations of chelating agent higher than required to chelate the available Mn^{2+} and Mg^{2+} might chelate the Zn^{2+}. However, by this stage the enzyme is already inactivated by lack of dissociable bivalent cation. Chelation of Zn^{2+} is a possible explanation of the failure totally to restore polymerase activity by addition of Mn^{2+} and Mg^{2+} to enzyme inhibited by the highest concentration of 8-hydroxyquinoline (Fig. 7).

Demonstration of the mode of action of an inhibitor in a simplified system *in vitro* provides a strong indication, though not complete proof, of the way it acts in the intact cell. We have shown that lomofungin and 8-hydroxyquinoline inhibit *E. coli* RNA polymerase *in vitro* by chelating Mn^{2+} and Mg^{2+}. Yeast RNA polymerases also require Mn^{2+} and Mg^{2+} (Ponta *et al.*, 1971; Brogt & Planta, 1972; Adman *et al.*, 1972) and lomofungin inhibits the activity of yeast RNA polymerases *in vitro* (Cano *et al.*, 1973). These data suggest that lomofungin and 8-hydroxyquinoline might inhibit RNA synthesis in intact yeast cells by chelation of the dissociable bivalent cations required for polymerase activity. There is experimental evidence which is consistent with chelation as the mechanism of action of the inhibitors *in vivo*. The ability to chelate falls with decreasing pH. When the internal pH of yeast cells was decreased by growing cells in culture medium at low pH, the inhibition of RNA synthesis by 8-hydroxyquinoline was also lessened (Fraser & Creanor, 1974).

Our suggestion that chelation is the mode of action of lomofungin and 8-hydroxyquinoline permits explanation of their effects on some other aspects of metabolism in yeast.

The synthesis of 5S RNA and tRNA is inhibited less than synthesis of rRNA and polydisperse RNA by 8-hydroxyquinoline (Fraser & Creanor, 1974) and lomofungin (Cano *et al.*, 1973; Fraser *et al.*, 1973; Cannon & Jimenez, 1974). This may be related to the different bivalent-cation requirements of different RNA polymerases. Yeast polymerase III has a lower total requirement for dissociable bivalent cations than polymerases I and II, and is fully active with Mg^{2+} alone, whereas polymerases I and II require Mn^{2+} as well as Mg^{2+} for full activity (Ponta *et al.*, 1972). The decrease in bivalent cation concentration, and particularly in Mn^{2+} concentration, likely with lomofungin or 8-hydroxyquinoline, should inhibit polymerase III less than polymerases I and II. This suggests that yeast polymerase III may be responsible for 5S RNA and tRNA synthesis. Polymerase III in animal cells, which also has a lower requirement for dissociable bivalent cations than polymerases I and II (Roeder & Rutter, 1969; Price & Penman, 1972), has been shown to synthesize 5S RNA and tRNA (Weinmann & Roeder, 1974).

We are unable to offer any explanation of the comparative resistance of synthesis of yeast 5S RNA and tRNA to inhibition by lomofungin based on primary inhibition of yeast RNA polymerases by chelation of the tightly bound Zn^{2+} (Pavletich *et al.*, 1974). Valenzuela *et al.* (1973) found that sea-urchin RNA polymerases I, II and III were inhibited to similar extents by the Zn^{2+} chelator *o*-phenanthroline.

Lomofungin and 8-hydroxyquinoline might be expected to inhibit any cellular process requiring bivalent cations. This is certainly true for DNA synthesis. 8-Hydroxyquinoline inhibits DNA synthesis in fission yeast (Fraser & Creanor, 1974). Cannon & Jimenez (1974) reported inhibition by lomofungin of DNA synthesis in budding yeast, and suggested that this might be caused by the especial sensitivity to lomofungin of RNA synthesis particularly involved in DNA synthesis. This suggestion is made unlikely by the observation by Pavletich *et al.* (1974) that DNA polymerase is directly inhibited *in vitro* by lomofungin.

Protein synthesis, which requires Mg^{2+}, is not immediately inhibited in yeast by lomofungin (Cano *et al.*, 1973; Cannon *et al.*, 1973; Fraser *et al.*, 1973) or by 8-hydroxyquinoline (Fraser & Creanor, 1974). However, in vertebrate cells both 8-hydroxyquinoline (Fraser & Creanor, 1974) and lomofungin (R. S. S. Fraser & J. Creanor, unpublished work) inhibit protein synthesis as rapidly as they inhibit RNA synthesis. These findings suggest that there is nothing intrinsic to lomofungin or 8-hydroxyquinoline which makes them selective inhibitors of nucleic acid synthesis in yeast. In an earlier paper (Fraser & Creanor, 1974), we suggested that the pH of yeast cytoplasm might be too low for significant chelation by 8-hydroxyquinoline. Under these circumstances protein synthesis would not be inhibited. There is evidence that the pH of the yeast nucleus may be higher than that of the cytoplasm (Conway & Downey, 1950) which would permit chelation in the nucleus and thus selective inhibition of RNA synthesis. In vertebrate cells the average internal pH is much higher than that of yeast (Caldwell, 1956). 8-Hydroxyquinoline and lomofungin would be expected to be capable of chelating in both nucleus and cytoplasm, thus inhibiting both RNA and protein synthesis.

The suggestion by Pavletich *et al.* (1974) that lomofungin inhibits RNA synthesis solely by chelating the Zn^{2+} tightly bound to the RNA polymerase might appear an attractive explanation of why lomofungin does not inhibit yeast protein synthesis. However, their model does not explain the rapid inhibition of protein synthesis in vertebrate cells (Fraser & Creanor, 1974) and does not allow for the ability of lomofungin to chelate Mg^{2+} as well as Zn^{2+} (Figs. 1 and 3).

This work was supported by the Medical Research Council and Science Research Council. We thank Professor J. M. Mitchison and Dr. M. E. Bindloss for useful discussions.

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Turnover of Polyadenylated Messenger RNA in Fission Yeast

Evidence for the Control of Protein Synthesis at the Translational Level

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(Received May 20/September 6, 1975)

Polyadenylated RNA was isolated from fission yeast (*Schizosaccharomyces pombe*) total RNA using oligo(dT)-cellulose, and was studied as a model for messenger RNA. The half-life of polyadenylated RNA was measured by two independent methods. (a) The rate of labelling of polyadenylated RNA during incubation of cells with [5-^{3}H]uridine was measured. A half-life of 40–45 min was found by comparing the experimental data with theoretical curves calculated for labelling of RNAs with various half-lives. The influence of precursor-pool specific activity on RNA labelling kinetics is considered. (b) Cells were labelled with [5-^{3}H]uridine then further RNA synthesis was inhibited by addition of 8-hydroxyquinoline. The rate of loss of radioactivity from polyadenylated RNA indicated a half-life of 50 min. The half-life found by these two methods is about one-third of the cell doubling time, and is much longer than previous estimates by indirect methods of yeast messenger RNA half-life. Both experimental methods provided evidence for the existence of two populations of polyadenylated RNA. The larger population has a half-life of 40–50 min; a much smaller population is probably turning over more rapidly.

After inhibition of RNA synthesis by 8-hydroxyquinoline, the rate of total protein synthesis declined much more rapidly than the polyadenylated RNA content of the cells. However, 60 min after inhibition of RNA synthesis there was a small rise in the rate of protein synthesis. These data are interpreted as evidence for mechanisms controlling protein synthesis which operate at the level of messenger RNA translation.

Previous estimates of the half-life ($t_{0.5}$) of messenger RNA in yeasts have been made by indirect methods. Values within the range 20–25 min have been obtained by inhibiting RNA synthesis, then following the decay of protein synthesis [1,2] or the disappearance of polyribosomes [3–5].

Recently it has been discovered that many mRNAs in eukaryotes, including yeasts, contain a polyadenylic acid sequence [6–10]. The development of methods for the separation of polyadenylated mRNA from total RNA [7,11] has made it possible to examine the turnover rate of a defined fraction of mRNA directly. This paper reports an investigation of the $t_{0.5}$ of polyadenylated mRNA in fission yeast.

Abbreviations. $t_{0.5}$, half-life time; poly(A), polyadenylic acid; oligo(dT), oligothymidylic acid; poly(A)$^+$ RNA, RNA containing a polyadenylic acid sequence; poly(A)$^-$ RNA, RNA lacking a polyadenylic acid sequence; butyl-PBD, 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole.

Enzymes. Ribonuclease (EC 3.1.4.22); acid phosphatase (EC 3.1.3.2); hexokinase (EC 2.7.1.1); sucrase (invertase) (EC 3.2.1.26); maltase (EC 3.2.1.20).

MATERIALS AND METHODS

Culture Conditions

Fission yeast (*Schizosaccharomyces pombe* Lindner), strain N.C.Y.C. 132, A.T.C.C. 24751, was grown at 32 °C in a minimal medium EMM 2 [12]. The mean generation time was 150 min. Cultures in the early part of the exponential growth phase, with cell concentrations of $(2.5–4.0)\times10^6$/ml, were used for experiments.

Radioisotopes

[5-^{3}H]Uridine (29 Ci/mmol) and [4,5-^{3}H]leucine (15 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, UK).

Uptake and Incorporation of [5-^{3}H]Uridine

Cells were incubated with 20 μCi/ml [5-^{3}H]uridine. The incorporation of radioactivity into RNA was meas-

ured by mixing 0.5-ml samples of culture with 5 ml ice-cold 5% CCl_3COOH. The cells were collected by filtration on Whatman GF/A glass-fibre paper and washed three times with 5 ml cold 5% CCl_3COOH. Acid-insoluble radioactivity on the dried filters was measured by counting in 5 ml 0.5% butyl-PBD/toluene scintillator in a Packard liquid-scintillation counter. Uridine uptake was measured by mixing 0.5 ml of culture with 5 ml ice-cold water containing 100 µg/ml non-radioactive uridine. The cells were collected by filtration, washed three times with 5 ml ice-cold water and total uridine uptake was determined by scintillation counting as above. The total radioactivity of the acid-soluble pool was found by subtracting incorporation from uptake.

Pulse-Chase Labelling of RNA

For studies of the rate of labelling of poly(A)$^+$ RNA, cells were incubated for various pulse times with 20 µCi/ml [5-^{3}H]uridine. To study the rate of decay of poly(A)$^+$ RNA radioactivity after inhibition of RNA synthesis, 6.25 µCi/ml [5-^{3}H]uridine was added to cultures for a pulse incubation of 8 min or 30 min. The cells were collected by filtration on an Oxoid 0.45-µm pore-size filter, and resuspended in the same volume of chase medium. The chase medium had been conditioned by growth of cells and their removal by filtration at a density of 2.5×10^6/ml. The chase medium lacked [^{3}H]uridine, but contained 100 µg/ml non-radioactive uridine and 50 µg/ml 8-hydroxyquinoline, a rapid and selective inhibitor of RNA synthesis in yeast [2, 13]. Collection and resuspension of cells took less than 1 min.

Extraction of RNA

Cells from 20–30 ml culture were collected by filtration on 0.45-µm pore-size Millipore filters, and resuspended in 5 ml ice-cold 2% sodium triisopropylnaphthalene sulphonate [14], 10 mM NaCl, 50 mM Tris-HCl, pH 7.8. 2 g glass beads (40 mesh) were added and the cells were broken in a Vibromixer (Shandon Scientific, London, U.K.) by agitation for 5 min at maximum setting. An equal volume of phenol mixture (1000 g phenol, 200 ml *m*-cresol, 1 g 8-hydroxyquinoline, 300 ml chloroform, 200 ml water) was added and deproteinisation was performed as described previously [15]. The extracted nucleic acids were precipitated by addition of 2.5 volumes ethanol and NaCl to 0.3 M. After 12 h at 0 °C, precipitated nucleic acids were collected by sedimentation at $2000 \times g$ for 5 min. The nucleic acids were further purified by redissolving in 2 ml 0.5% sodium dodecylsulphate, 0.15 M sodium acetate, pH 6.0, and reprecipitation with 2.5 volumes ethanol at 0 °C for 12 h. After three such cycles of reprecipitation, the nucleic acids were stored as a suspension in 80% ethanol, 0.1% sodium dodecylsulphate, 0.03 M sodium acetate, pH 6.0, at 0 °C.

To determine the total RNA extracted, a portion of the nucleic acid suspension was sedimented and hydrolysed in 0.5 N $HClO_4$ at 70 °C for 20 min. The ultraviolet absorption spectrum of the hydrolysate was measured in the Unicam SP800 recording spectrophotometer. RNA concentration was calculated using a molar absorption value at 260 nm of $12400\ M^{-1} \times cm^{-1}$, calculated for a solution of equal molar quantities of the four ribonucleotides at acid pH. DNA is only 1% of total nucleic acid in fission yeast [12] and thus did not significantly interfere in this RNA assay.

Oligo(dT)-Cellulose Fractionation of RNA

Radioactive yeast RNA was separated into RNA containing a poly(A) sequence [poly(A)$^+$ RNA] and RNA lacking a poly(A) sequence [poly(A)$^-$ RNA] using the binding, at high salt concentrations, of the poly(A) sequences to the complementary homopolynucleotide, oligo(dT), immobilised on cellulose [7, 11]. Between 50 µg and 200 µg total RNA was dissolved in 1 ml binding buffer (400 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.8, 0.2% sodium dodecylsulphate) and shaken gently for 30–60 min at 20 °C with 20 mg oligo(dT)-cellulose (Collaborative Research Inc., Waltham, Mass., U.S.A.; binding capacity 42.8 A_{260} units of poly(A)/g). The cellulose was sedimented at $12000 \times g$ for 1 min, then washed four times with 0.5 ml binding buffer. The binding buffer supernatants were combined and the poly(A)$^-$ RNA precipitated by 2.5 volumes ethanol at −20 °C for 12 h. To determine the total radioactivity of the poly(A)$^-$ RNA, the precipitate was collected by filtration on glass-fibre paper, washed three times with 5 ml cold 5% CCl_3COOH and ^{3}H radioactivity on the filter was determined as above.

The cellulose was then washed four times with 0.5 ml elution buffer (binding buffer minus NaCl) to release the poly(A)$^+$ RNA. For sucrose gradient analysis of the poly(A)$^+$ RNA, NaCl to a final concentration of 0.3 M, 50 µg non-radioactive yeast carrier RNA and 2.5 volumes ethanol were added to the combined elution buffer washes, and precipitated RNA was collected by centrifugation after 12 h at −20 °C. To estimate the total radioactivity of the poly(A)$^+$ RNA, the combined elution buffer washes were dried in scintillation vials at 70 °C, hydrolysed in 0.3 ml 0.5 N $HClO_4$ for 20 min at 70 °C, then mixed with 7 ml scintillator (0.5% butyl-PBD, 40% 2-methoxyethanol, 60% toluene).

The total recovery of radioactivity from the oligo(dT)-cellulose was over 95% of the input. Over 90% of the radioactivity in the poly(A)$^+$ RNA frac-

tion again bound to oligo(dT)-cellulose at high salt concentration when the poly(A)$^+$ RNA was reprocessed with fresh cellulose. No significant amount of radioactivity bound to the cellulose at high salt concentration when the poly(A)$^-$ RNA fraction was reprocessed with fresh cellulose.

Sucrose Gradient Fractionation of RNA

Samples of up to 50 µg RNA were dissolved in 0.1 ml gradient buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 0.5% sodium dodecylsulphate) and layered on to 5-ml gradients of 5–25% sucrose in gradient buffer. The gradients were centrifuged for 1.75 h at 50000 rev./min ($234000 \times g$ at r_{av}) in a Spinco SW 50.1 rotor at 25 °C. 36–38 12-drop fractions were collected from each gradient. RNA was precipitated by addition of 200 µg bovine serum albumin as carrier and 0.5 ml ice-cold 10% CCl_3COOH. The precipitate was collected by filtration on glass-fibre paper, washed three times with 5 ml cold 5% CCl_3COOH and ^3H radioactivity was determined by counting in butyl-PBD toluene scintillator.

Determination of Specific Activity of 18-S rRNA

About 20 µg [^{3}H]uridine-labelled RNA was fractionated on a 2.4% polyacrylamide gel [16] for 2.5 h at 5 mA/gel, 8 V/cm gel length. Gels were scanned for absorbance at 265 nm in a Joyce-Loebl gel scanner, frozen in solid CO_2 and sliced transversely at 1-mm intervals. The slices were incubated with 0.3 ml 0.5 N $HClO_4$ at 70 °C for 20 min, mixed with 7 ml butyl-PBD/toluene/methoxyethanol scintillator and ^3H radioactivity was measured. Fig. 1 shows a typical absorbance and radioactivity profile. The weight of 18-S rRNA was obtained from its peak area on the ultraviolet absorption scan; peak area is linearly proportional to the weight of RNA in the peak [17]. Radioactivity in the 18-S rRNA was calculated by addition of the radioactivities of the individual gel slices in the 18-S radioactivity peak, after subtracting the polydisperse background radioactivity on the gel.

Protein Synthesis

The rate of protein synthesis was measured by transferring 1-ml samples of culture to prewarmed tubes containing 10 µCi [4,5-^{3}H]leucine and 1 µg non-radioactive leucine. The samples were incubated for 4 min at 32 °C, then added to 5 ml ice-cold 5% CCl_3COOH containing 50 µg/ml non-radioactive leucine. The cells were collected by filtration, washed three times with 5 ml 5% CCl_3COOH and acid-insoluble radioactivity on the filters was measured by counting in butyl-PBD/toluene scintillator. The contribution to cold-acid-insoluble radioactivity of [^{3}H]-leucinyl tRNA, measured by the loss of insoluble radioactivity after 15 min at 90 °C in 5% CCl_3COOH [18], was insignificant.

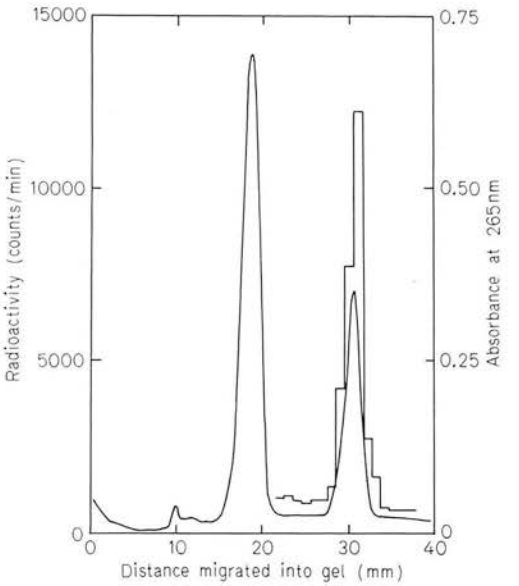

Fig. 1. *Determination of the specific activity of yeast 18-S rRNA.* Polyacrylamide gel fractionation of [^{3}H]uridine-labelled RNA from yeast, labelled for 80 min with 20 µCi/ml [5-^{3}H]uridine. The continuous line shows absorbance at 265 nm, the histogram ^3H radioactivity. The 25-S rRNA is at 19 mm, the 18-S rRNA at 31 mm

RESULTS AND DISCUSSION

Characterization of Poly(A)$^+$ RNA

Fig. 2A shows sucrose gradient fractionations of typical poly(A)$^+$ and poly(A)$^-$ RNA fractions. The poly(A)$^+$ RNA was polydisperse, sedimenting in the range from 6 S to over 30 S, with a broad peak at 11 S. There was no sign of significant contamination of the poly(A)$^+$ RNA by labelled tRNA or rRNA. The poly(A)$^-$ RNA contained radioactive tRNA, 18-S and 25-S rRNA and some polydisperse labelled RNA which did not bind to the oligo(dT)-cellulose at high salt concentrations.

The poly(A)$^+$ RNA contains a nucleotide sequence with very high adenine content and a length within the range 25–75 nucleotide residues. This sequence is resistant to degradation by pancreatic and T_1 ribonuclease. No such sequence is found in the poly(A)$^-$ RNA. (R. S. S. Fraser, unpublished results). Under the conditions used for binding of poly(A)$^+$ RNA to oligo(dT)-cellulose, poly(A) sequences with lengths down to a lower limit of 10–15 residues should be bound [38]. It is thus unlikely that the oligo(dT)-cellulose failed to bind any significant amount of the poly(A)$^+$ RNA.

Poly(A)$^+$ RNA was around 5–25% of the total RNA labelled by a pulse of [^{3}H]uridine; the percentage

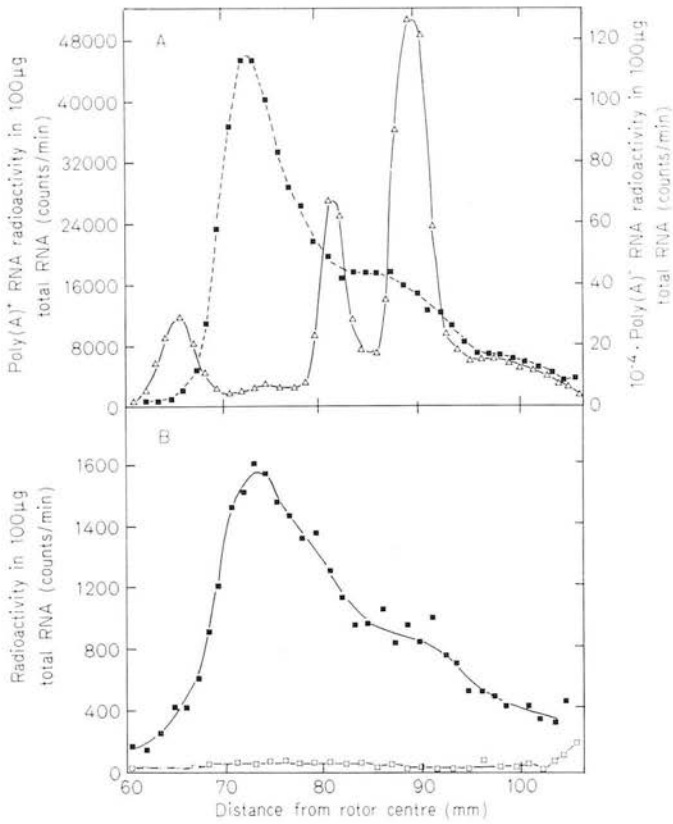

Fig. 2. *Sucrose gradient sedimentation of [^{3}H]uridine-labelled yeast RNA.* (A) Poly(A)$^-$ RNA (△——△) and poly(A)$^+$ RNA (■——■) from yeast labelled for 80 min with 20 µCi/ml [5-^{3}H]uridine. (B) (■——■) Poly(A)$^+$ RNA from cells labelled for 30 min with 6.25 µCi/ml [5-^{3}H]uridine, then resuspended for 150 min in non-radioactive culture medium containing 100 µg/ml unlabelled uridine and 50 µg/ml 8-hydroxyquinoline. (□——□) Poly(A)$^+$ RNA from cells resuspended in culture medium with 50 µg/ml 8-hydroxyquinoline and labelled with 6.25 µCi/ml [5-^{3}H]uridine from 120–150 min after resuspension. All radioactivity values are expressed per 100 µg total RNA fractionated on oligo(dT)-cellulose.

of radioactivity in the poly(A)$^+$ RNA decreased with increasing length of pulse incubation.

In this investigation poly(A)$^+$ RNA has been studied as a model for the mRNA metabolism of the cell. It is important to ask whether the poly(A)$^+$ RNA is in fact mRNA, and what proportion of the cellular mRNA it accounts for. The poly(A)$^+$ RNA isolated shows several properties characteristic of mRNA. It is heterogeneous in size; the actual size range corresponds well to the sizes of mRNAs which would be required to code for a reasonable distribution of protein sizes. The poly(A)$^+$ RNA was labelled to a very high specific activity in a pulse incubation. It was not possible to calculate the exact specific activity, as the weight of poly(A)$^+$ RNA prepared by oligo(dT)-cellulose fractionation was too low to measure accurately. The presence of a poly(A) sequence is a characteristic of many eukaryotic mRNAs [6–10]. Finally, after a 1-h pulse incubation, about 70% of the radioactive poly(A)$^+$ RNA in the cell is associated with the polyribosome fraction (R. S. S. Fraser, unpublished results). From these criteria it is concluded that the poly(A)$^+$ RNA is mRNA. The possibility cannot be excluded that some of the poly(A)$^+$ RNA is not mRNA, though no functions for poly(A)$^+$ RNA other than those of mRNA or mRNA precursor have been described.

The poly(A)$^+$ RNA most probably does not represent the total mRNA content of the cell, as at least some of the non-polyadenylated, polydisperse RNA (Fig. 2A) might have mRNA activity. In budding yeast, McLaughlin *et al.* [9] found that some of the polyribosome-associated polydisperse RNA did not contain a poly(A) sequence. Shiokawa and Pogo [19], however, found the non-polyadenylated polydisperse RNA of budding yeast mainly in the nuclear and membrane fractions. mRNAs lacking a poly(A) sequence have been found in other eukaryotes [20–22].

It is difficult to estimate exactly the amount of poly(A)$^-$ polydisperse RNA, as it cannot be separated from the other poly(A)$^-$ RNAs. Subjective estimates from sedimentation profiles of poly(A)$^-$ and poly(A)$^+$ RNAs suggest that the poly(A)$^+$ RNA in fission yeast represents about half of the total labelled polydisperse RNA. Thus poly(A)$^+$ RNA is likely to represent a major fraction of yeast mRNA, and provides a suitable model for study of yeast mRNA metabolism. The half-life of the poly(A)$^+$ RNA has been measured by two methods.

Measurement of Poly(A)$^+$ RNA Half-Life by the Kinetics of Labelling

Greenberg [23] derived Eqn (1) for the labelling of an RNA species during the approach to steady state:

$$A/A_\infty = 1 - e^{-\ln 2\left(\frac{1}{t_d} + \frac{1}{t_{0.5}}\right)t} \qquad (1)$$

where A is the specific activity of the RNA after labelling time t; A_∞ is the specific activity as $t \to \infty$; t_d is the cell doubling time and $t_{0.5}$ is the half-life of the RNA. The equation holds for cells in exponential growth, when the RNA is labelled from a precursor pool of constant specific activity.

The experimental approach is to label cells for different times, prepare poly(A)$^+$ RNA and determine its specific activity. These experimental data are then compared with a series of theoretical curves for change in A/A_∞ with time, calculated from Eqn (1) for RNAs with different $t_{0.5}$ values.

Fig. 3A shows the change in specific activity of poly(A)$^+$ RNA during a pulse-labelling with [^{3}H]-uridine. As it was not possible to measure the weight of poly(A)$^+$ RNA prepared, the specific activity was calculated as radioactivity in poly(A)$^+$ RNA per unit

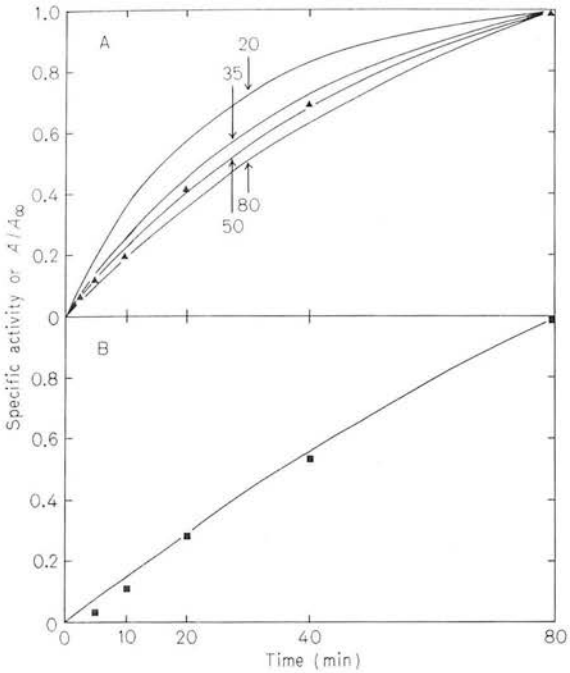

Fig. 3. *Determination of the half-life of poly(A)$^+$ RNA by the kinetics of labelling from a precursor pool of constant specific activity.* (A) (▲) Experimentally determined changes in poly(A)$^+$ RNA specific activity with length of pulse incubation with 20 μCi/ml [5-^{3}H]uridine. Each point is the mean of six replicate determinations. Standard errors of the means were within the physical dimensions of the symbols. The continuous lines show theoretical labelling curves (A/A_∞) for RNAs with half-lives of 20, 35, 50 and 80 min. (B) (■) Experimentally determined changes in 18-S rRNA specific activity with length of pulse. The continuous line shows the theoretical change in A/A_∞ for an RNA with infinite half-life. All experimental data have been normalized to a value of 1.0 at 80 min to facilitate comparison of curves

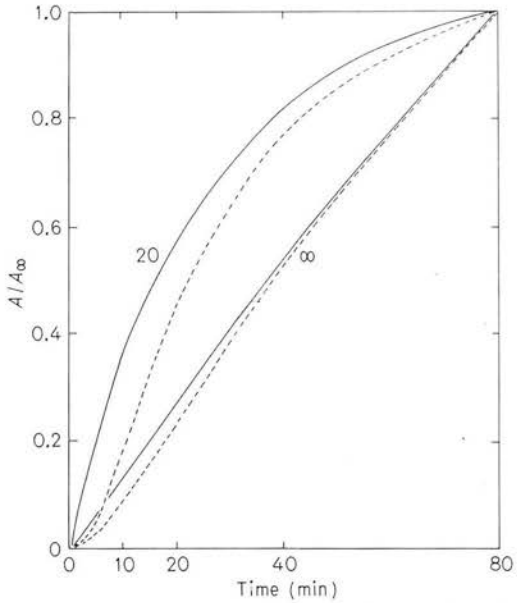

Fig. 4. *Theoretical curves for changes in* A/A_∞ *with pulse time of RNAs with half-lives of 20 min and infinity.* (——) Labelling from a precursor pool of constant specific activity, calculated from Eqn (1) [23]. (– – –) Labelling from a precursor pool, the specific activity of which rose linearly from 0 to 1 during the first 10 min, then remained at 1 for the remainder of the pulse. The curves were calculated from Eqn (1) and corrected for changes in precursor specific activity during the labelling period

weight of total RNA fractionated on oligo(dT)-cellulose. This makes the reasonable assumption that poly(A)$^+$ RNA is a constant proportion of the mass of total RNA throughout the pulse incubation. The experimental data and theoretical curves for labelling of RNAs with different $t_{0.5}$ values have been normalized to a value of 1.0 at 80 min to facilitate comparisons. It is clear that the theoretical curve which best fits the experimental data is that for an RNA with $t_{0.5}$ of 50 min.

Before accepting this estimate of mRNA $t_{0.5}$, it is necessary to question whether the requirement of labelling from a precursor pool of constant specific activity was satisfied. Obviously the pool specific activity is zero at the start of the pulse and there must be some delay before constant specific activity is reached. A test is required of whether this deviation from constant specific activity is sufficient to make Eqn (1) inapplicable to the labelling of the RNA.

For this test, Greenberg [23] examined the labelling of rRNA. In this case the $t_{0.5}$ may be taken as approaching infinity. If, therefore, the experimental points for labelling of rRNA during a pulse incubation follow the theoretical curve for labelling of an RNA with $t_{0.5} = \infty$ it can be concluded that labelling was from a precursor pool of essentially constant specific activity. Fig. 3 B shows changes in the specific activity of 18-S rRNA during a pulse incubation. It is clear that the experimental points do fit closely the theoretical curve for $t_{0.5} = \infty$. However, with very short pulse times the experimental points lie a little below the theoretical curve. This could be due to a short delay before the precursor pool reaches constant specific activity, but part of the divergence must be due to the delay in appearance of 18-S rRNA while the ribosomal RNA precursor is processed to the mature forms [24].

A criticism of the use of stable RNA labelling as a test of precursor-pool constant specific activity is that the labelling pattern of stable RNA is relatively insensitive to small changes in specific activity occurring early in the pulse. The labelling pattern of rapidly turning-over RNA is more likely to be distorted by small early changes in specific activity. This is illustrated in Fig. 4, which shows theoretical curves for labelling of RNAs with 20-min and infinite half-lives, either from a pool of constant specific activity, or from a pool in which the specific activity rose for 10 min then became constant. It is clear that the introduction of the changing precursor specific activity factor has caused less divergence between the two $t_{0.5}$ = infinity curves than between the two $t_{0.5}$ = 20 min curves. Thus close agreement between

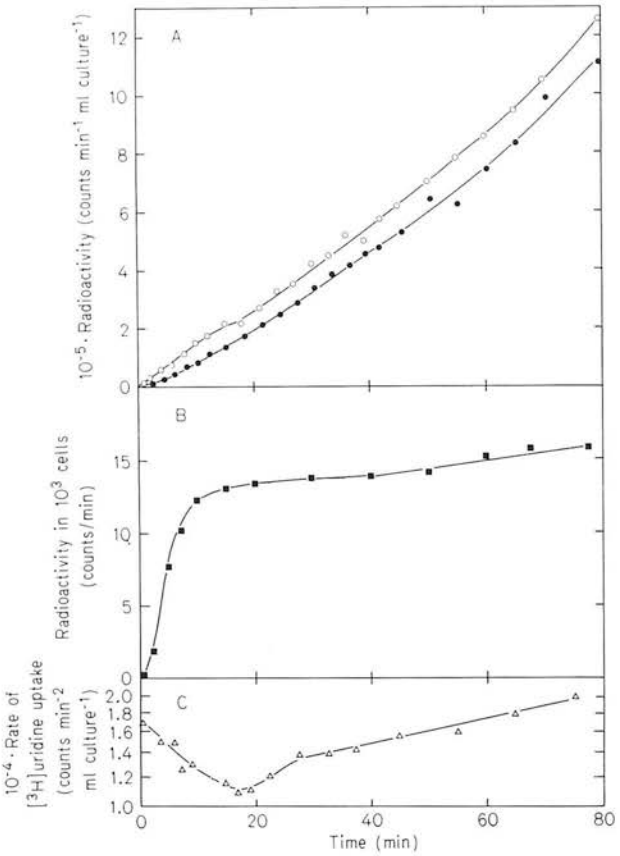

Fig. 5. *Uptake and incorporation of [5-^{3}H]uridine by yeast, and changes in acid-soluble pool radioactivity and in the rate of uptake of uridine.* (A) [^{3}H]Uridine uptake (○) and incorporation (●)/ml culture for cells grown with 20 μCi/ml [5-^{3}H]uridine. (B) Acid-soluble pool radioactivity/thousand cells, calculated from (A) for a cell doubling time of 150 min. (C) Changes in the rate of [^{3}H]uridine uptake/ml culture. Data derived from (A)

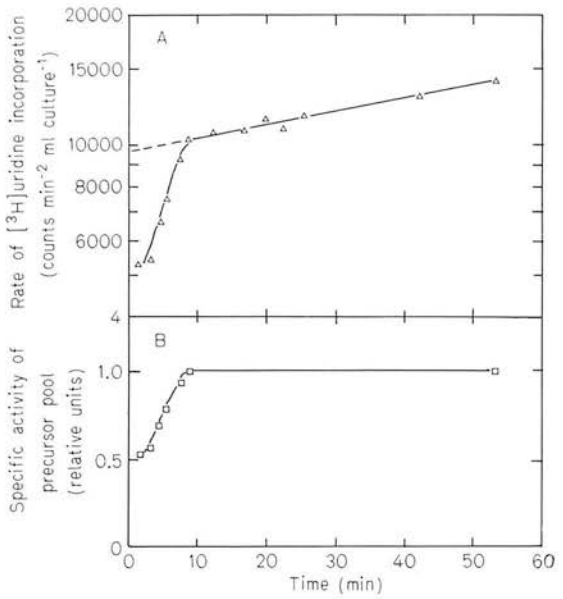

Fig. 6. *Changes in the rate of incorporation of [5-^{3}H]uridine into RNA (A) and calculated changes in specific activity of the precursor pool (B) during a pulse incubation.* Rates of incorporation were derived from Fig. 5A. (----) Shows the extrapolation of the exponential rise in incorporation rate. Pool specific activity was calculated from the incorporation rate data, as the experimentally determined rate of incorporation divided by the level of the exponential line or its extrapolation at that time

theoretical and experimental labelling data for 18-S rRNA (Fig. 3B) does not really provide adequate evidence of an essentially constant specific activity precursor pool. It was, therefore, necessary to investigate the pattern of change in specific activity of the precursor pool in more detail. Direct examination of changes in pool specific activity during the pulse was rejected, as any compartmentalisation of the pool, or the existence of a 'by-pass pool' [25] would make the results meaningless for interpreting rates of RNA synthesis.

Instead, the total radioactivity of the acid-soluble pool was measured, by subtracting radioactivity incorporated into RNA from total uptake of radioactivity (Fig. 5A). The acid-soluble pool radioactivity per thousand cells rose sharply for the first 10 min of the pulse, then remained nearly constant for the remainder of the incubation (Fig. 5B). Examination of the rate of uridine uptake during the pulse (Fig. 5C) showed an initially high rate of uptake, which fell during the first 20 min. An exponential rise in the rate of uptake/ml culture, which is the pattern expected in an exponentially growing culture, did not commence until 30 min. The initially high rate of uridine uptake is consistent with an expansion of the uridine and connected pools when cells are supplied with exogenous uridine. Under these conditions the specific activity of the precursor pool would approach a constant value faster than would the total radioactivity of the pool (Fig. 5B). These data merely suggest that the precursor pool quickly attained constant specific activity, and confirm that no depletion of isotope occurred towards the end of the pulse. They do not establish when constant specific activity was reached, or permit calculation of mRNA labelling curves based on the pattern of precursor specific activity.

An alternative approach to study of the specific activity of the immediate precursor pool is to consider changes in the rate of incorporation of radioactivity into RNA. When this rises exponentially in an exponentially growing culture, the precursor specific activity has become constant. Furthermore, by examining RNA labelling, errors arising from pool compartmentalisation and 'by-pass pools' are avoided. Fig. 6A shows that the rate of incorporation of uridine into RNA became linear, on a logarithmic plot, at 7.5 min. The slope of the line corresponds to a doubling in the rate of incorporation over one cell-generation time. The straight line was extrapolated to zero time, and precursor specific activity was

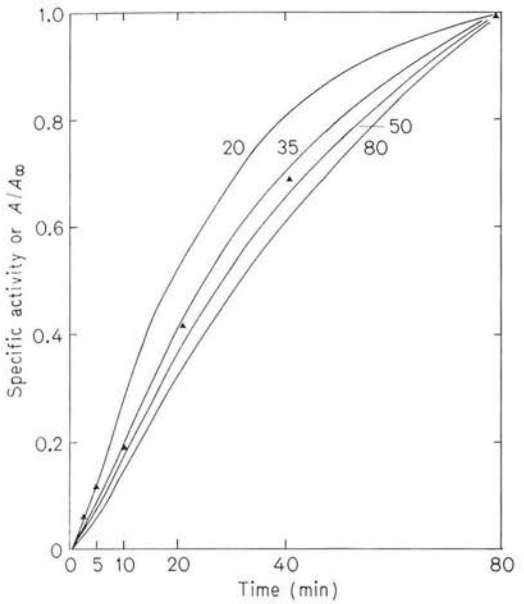

Fig. 7. *Determination of the half-life of poly(A)$^+$ RNA by labelling from a precursor pool of changing specific activity.* (▲) Experimentally determined changes in poly(A)$^+$ RNA specific activity with length of pulse (data from Fig. 3A). The continuous lines show theoretical curves for changes in A/A_∞ of RNAs with half-lives of 20, 35, 50 and 80 min, labelled from a precursor pool having the pattern of changing specific activity shown in Fig. 6B

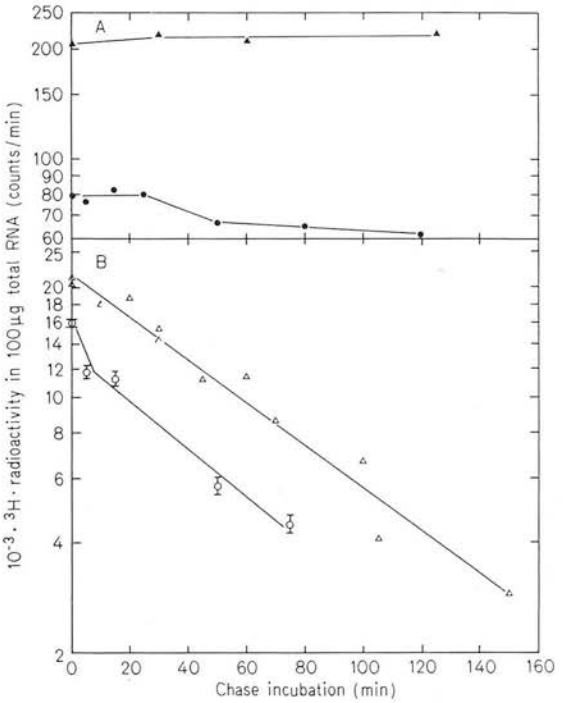

Fig. 8. *Changes in specific activity of (A) poly(A)$^-$ RNA and (B) poly(A)$^+$ RNA during a non-radioactive chase incubation with 8-hydroxyquinoline.* Cells were labelled with 6.25 µCi/ml [5-^{3}H]uridine for 8 min (●, ○) or 30 min (▲, △) then resuspended (time 0) in non-radioactive culture medium containing 100 µg/ml unlabelled uridine and 50 µg/ml 8-hydroxyquinoline. Changes in specific activity during the chase are expressed per 100 µg total RNA fractionated on oligo(dT)-cellulose. Each point is the mean of 3 (▲, ●, △) or 4 (○) determinations. The standard error of the mean is shown by vertical bars for points (○). The linear regression coefficient for the logarithm of poly(A)$^+$ RNA specific activity on time for points shown (△) is −0.983

expressed as the measured rate of incorporation divided by the level of the straight line at that time (Fig. 6B).

It is now possible to calculate theoretical curves for labelling of RNAs from a precursor pool showing this pattern of change in specific activity; these are compared with experimental data for labelling of poly(A)$^+$ RNA in Fig. 7. From 10 min, all the experimental points lie between the theoretical curves for 35-min and 50-min half-lives, indicating a $t_{0.5}$ of 40−45 min for the poly(A)$^+$ RNA. This is slightly shorter than the 50 min estimated from theoretical curves uncorrected for changes in precursor specific activity (Fig. 3A). In the first 10 min of the pulse (Fig. 7), the experimental points fell on the theoretical line for $t_{0.5}$ = 20 min. A possible explanation of this is that the poly(A)$^+$ RNA consists of two populations, a small, rapidly turning over one and a larger one turning over more slowly. The small population will account for most of the labelling within very short pulse times, the larger population will account for proportionally more of the labelling with increasing pulse times. Further support for this explanation will be presented below.

Measurement of Poly(A)$^+$ RNA Half-Life Using an Inhibitor of RNA Synthesis

Cells were incubated for 8 min or 30 min with [^{3}H]uridine, then transferred to non-radioactive 'chase' medium containing 8-hydroxyquinoline, an inhibitor of RNA synthesis. Explanations of the mode of action of 8-hydroxyquinoline, and evidence for its selectivity as an inhibitor of RNA synthesis in yeast are given elsewhere [2,13]. RNA was prepared from cells after various lengths of chase incubation. During the chase, no further incorporation of radioactivity into the poly(A)$^-$ RNA was detectable (Fig. 8A). The poly(A)$^-$ RNA consists mainly of stable tRNA and rRNA (Fig. 2A). Its relatively constant specific activity during the chase confirms the effectiveness of 8-hydroxyquinoline and resuspension in non-radioactive medium in stopping further RNA synthesis.

The $t_{0.5}$ of poly(A)$^+$ RNA was found from the rate of loss of radioactivity from this fraction during the chase (Fig. 8B). After a 30-min pulse incubation, the decay of poly(A)$^+$ RNA radioactivity followed first-order-type kinetics, with a $t_{0.5}$ of 50 min. This value is in good agreement with the estimate of 40−45 min from the labelling studies (Fig. 7).

The sedimentation profile of the poly(A)$^+$ RNA remaining after 150 min chase with 8-hydroxyquinoline is shown in Fig. 2B. The radioactivity is poly-

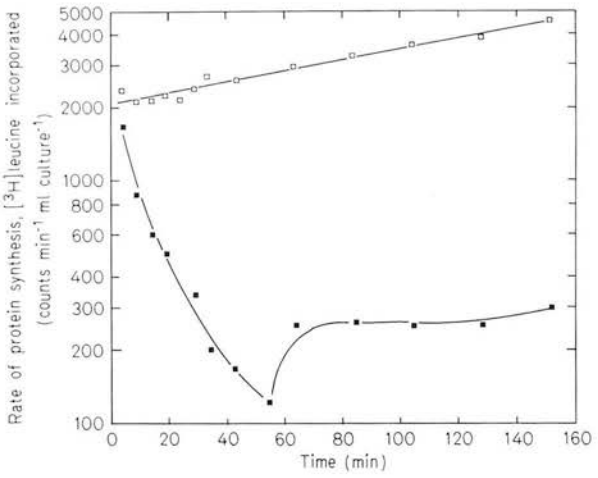

Fig. 9. *Changes in the rate of total protein synthesis after addition of 8-hydroxyquinoline to yeast cultures.* Cells were resuspended in medium containing 100 µg/ml uridine (□) or 100 µg/ml uridine and 50 µg/ml 8-hydroxyquinoline (■). The rate of protein synthesis was measured at various times after resuspension by incubating 1-ml samples of culture for 4 min with 10 µCi [^{3}H]leucine (specific activity adjusted to 10 µCi/µg with non-radioactive leucine)

disperse, from 6 S to over 30 S. This result excludes the possibility that any significant amount of the polyadenylated RNA which persisted during the chase was present as broken-down fragments of mRNA. A slight shortening of the average length of the poly(A) sequence was found to occur during the chase incubation with 8-hydroxyquinoline (R. S. S. Fraser, unpublished results). However, this decrease was too small to cause any significant reduction in binding of poly(A)$^+$ RNA to oligo(dT)-cellulose, and cannot have affected the half-life calculation.

As a check on the extent to which synthesis of poly(A)$^+$ RNA during the 8-hydroxyquinoline chase might have contributed to the total radioactivity of the poly(A)$^+$ RNA present, non-radioactive cells were collected and resuspended in chase medium containing 8-hydroxyquinoline. From 120–150 min of the chase, [^{3}H]uridine was added and RNA was extracted at 150 min. Fig 2B shows that no significant incorporation of radioactivity into the poly(A)$^+$ RNA was detectable.

When the decay of poly(A)$^+$ RNA radioactivity during an 8-hydroxyquinoline chase was examined in cells previously exposed to an 8-min pulse of [^{3}H]-uridine, the decay curve did not show simple first-order-type kinetics (Fig. 8B). Instead, there was a very rapid loss of radioactivity in the first 5 min of the chase. Thereafter, the poly(A)$^+$ RNA radioactivity decreased at a slower rate, corresponding to a $t_{0.5}$ of 50 min. These data confirm the suggestion arising from Fig. 7, that the poly(A)$^+$ RNA of the cells consists of two populations. After a pulse of 30 min, most of the radioactivity should be in the larger, longer-$t_{0.5}$ population, and the decay kinetics will reflect loss of radioactivity from this population. With the shorter pulse time, more of the radioactivity should be in the small, short-$t_{0.5}$ population, and the decay kinetics will reflect loss of radioactivity from both populations.

There has recently been a report that mammalian cells also contain a small, rapidly turning-over population of poly(A)$^+$ RNA, and a larger population with much longer average $t_{0.5}$ [26].

The possibility cannot be excluded that the rapidly turning-over fraction of yeast poly(A)$^+$ RNA is not a separate group of molecules, but consists of rapidly degraded segments of the longer-lived type of poly(A)$^+$ RNA. This could occur in the rapid processing of mRNA precursors to smaller, more stable mature mRNAs. Shiokawa and Pogo [19] reported that budding yeast nuclear poly(A)$^+$ RNA is about 10% larger than the cytoplasmic poly(A)$^+$ RNA.

Changes in the Rate of Protein Synthesis after Inhibition of RNA Synthesis

Changes in the rate of protein synthesis were followed after resuspending cells in normal culture medium or in medium containing 8-hydroxyquinoline to inhibit RNA synthesis. Control cultures showed an exponential rise in the rate of protein synthesis (Fig. 9). In cultures treated with 8-hydroxyquinoline a consistent pattern of changes was observed in replicate experiments. For the first 40–50 min after resuspension, the rate of protein synthesis decayed rapidly. The decay curve did not show simple first-order-type kinetics; the $t_{0.5}$ varied between 12 min and 20 min. By 50 min after resuspension the rate had fallen to about 5% of the control value. However, at 50–60 min, there was a reproducible rise in the rate of protein synthesis. Thereafter, the rate in hydroxyquinoline-treated cells was steady or rose very slowly.

In the accompanying paper Creanor *et al.* [27] describe changes in the activities of certain enzymes after addition of 8-hydroxyquinoline to fission yeast cultures. These changes in enzyme activity are consistent with the various effects of 8-hydroxyquinoline on the rate of total protein synthesis. The increase in activity of certain enzymes, such as derepressed acid phosphatase or hexokinase, is rapidly inhibited after addition of 8-hydroxyquinoline; this parallels the rapid fall in the rate of total protein synthesis in the first 40 min after addition of 8-hydroxyquinoline (Fig. 9). With basal sucrase and maltase, Creanor *et al.* [27] found that the activities remained the same as in control cultures for about 60 min after addition of 8-hydroxyquinoline, and then increased more rapidly than in control cultures. The commencement of this 'superproduction' of enzyme, which depended on protein synthesis rather than on activation of

existing enzyme [27] coincided with the rise in the rate of total protein synthesis found at 50–60 min (Fig. 9).

It should be noted that in no case has any recovery in the rate of RNA synthesis been found in cells after long exposure to 8-hydroxyquinoline. Therefore the rise in the rate of total protein synthesis after 50–60 min (Fig. 9) and the onset of 'superproduction' of particular enzymes cannot be explained by a relaxation of the inhibition of mRNA synthesis.

The ability of cells to increase the rate of total protein synthesis after 50–60 min, and to synthesise large amounts of sucrase after this time [27] serves as an internal control indicating that the rapid inhibition of total protein synthesis after addition of 8-hydroxyquinoline (Fig. 9) was not caused by any direct effect of 8-hydroxyquinoline on protein synthesis.

Suggested Mechanisms for Control of Protein Synthesis

The effects of 8-hydroxyquinoline on mRNA decay, total protein synthesis and enzyme synthesis [27] suggest that there may be mechanisms for control of protein synthesis operating at the translational level in fission yeast. It is clear that the rate of protein synthesis (Fig. 9) declines much faster than the presumed mRNA content of the cell (Fig. 8 B) after addition of 8-hydroxyquinoline. A similar effect has been found in mammalian cells after addition of actinomycin D [23,28,29]. Singer and Penman [28] postulated that synthesis of an unstable RNA, which is sensitive to actinomycin D, is required for translation of the relatively stable mRNA. Clearly such a mechanism of translational control would also account for the effects of 8-hydroxyquinoline on yeast protein synthesis and mRNA turnover. Small RNA molecules, which may act as promoters of translation, have been reported in mammalian cells [30,31].

A mechanism of control operating at the translational level may also be postulated to explain the rise in the rate of protein synthesis after 50–60 min 8-hydroxyquinoline treatment (Fig. 9) and the simultaneous onset of 'superproduction' of basal sucrase and maltase [27]. This mechanism involves either an unstable control RNA, or an unstable control protein produced from an unstable RNA. The mechanism envisages a pool of the control molecule in normal cells, which prevents translation of some of the messenger. On addition of the 8-hydroxyquinoline the inhibitor pool begins to decay, until after 50–60 min there is insufficient to inhibit translation of these comparatively stable mRNAs, and protein synthesis rises, and 'superproduction' of sucrase and maltase begins. The fact that there is only a small rise in the rate of total protein synthesis at 50–60 min suggests that the amount of mRNA under such translational control may be a small percentage of the total. Furthermore, the observation by Creanor *et al.* [27] that basal sucrase and maltase were 'superproduced', whereas six other enzyme systems tested did not show 'superproduction', suggests that this proposed mechanism for inhibition of translation is specific for particular mRNAs. There are many reports of factors, both RNA and protein, which appear to inhibit translation of particular types of mRNA in mammalian cells [31–37].

Conclusion

The experiments described have shown, by two independent methods, that in fission yeast the average half-life of the poly(A)$^+$ RNA is 40–50 min. There is probably also a minor fraction of poly(A)$^+$ RNA which turns over more rapidly. The poly(A)$^+$ RNA probably represents a major fraction of cellular mRNA. The $t_{0.5}$ value of 40–50 min is considerably longer than yeast mRNA $t_{0.5}$ values derived indirectly by following the decay of protein synthesis [1,2] or polyribosomes [3–5] after inhibition of RNA synthesis.

The mRNA fraction studied in this investigation was prepared from total cell RNA; the picture of mRNA stability gained is thus an overall one, and is likely to include precursors to mRNA. It will be interesting to study the decay of poly(A)$^+$ RNA in subcellular fractions after 8-hydroxyquinoline treatment, to discover whether the poly(A)$^+$ RNA which persists after the decay of protein synthesis is present as precursor-type mRNA in the nucleus, or in some 'stored' form in the cytoplasm. Shiokawa and Pogo [19] found in budding yeast that poly(A)$^+$ RNA accumulated in the nucleus after inhibition of initiation of protein synthesis.

Comparison of the rate of turnover of poly(A)$^+$ RNA, the changes in rate of protein synthesis after addition of 8-hydroxyquinoline, and the effects of 8-hydroxyquinoline on enzyme synthesis reported by Creanor *et al.* [27] strongly suggest that much of the yeast protein synthesis may be under translational control. Furthermore, there appear to be two types of translational control mechanism, one promoting translation, the other inhibiting translation, probably of specific mRNAs. These data do not exclude the possibility that synthesis of some proteins could be regulated solely at the transcriptional level.

I thank Drs J. Creanor and J. W. May, and Prof. J. M. Mitchison for interesting discussions. This work was supported by a Grant from the Medical Research Council.

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Reprinted from *J. Mol. Biol.* (1976) **104**, 223–242

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Synthesis of Polyadenylated messenger RNA During the Cell Cycle of *Saccharomyces cerevisiae*

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(Received 16 June, and in revised form 29 January 1976)

The rates of ribosomal and polyadenylated messenger RNA synthesis were measured through the cell cycle of the budding yeast *Saccharomyces cerevisiae*. An exponentially growing culture was pulse-labelled with [2-^{3}H]adenine for 0·1 generation time, then fractionated according to cell volume by zonal centrifugation. A control experiment with a synchronously dividing culture showed that zonal fractionation corresponded to a fractionation by stage in the cell cycle.

Nucleic acids were extracted from each fraction of the cell cycle from the zonal rotor, and DNA, ribosomal and polyadenylated messenger RNAs were isolated. DNA synthesis occurred between 0·4 and 0·8 of the cell cycle as fractionated on the zonal rotor. The rate of ribosomal RNA synthesis per cell was constant during G_1‡, doubled early in the period of DNA synthesis, then remained constant at the new level for the remainder of the cell cycle. The rate of processing of ribosomal RNA precursors to mature ribosomal RNAs declined at the time of the increase in rate of ribosomal RNA synthesis, but recovered later in the cycle. This could mean that the pool of ribosomal proteins is small, and that the rate of ribosomal protein synthesis increases later in the cell cycle than the rate of ribosomal RNA synthesis. The rate of synthesis of polyadenylated messenger RNA was constant during G_1 and the earliest part of the period of DNA synthesis, then doubled over the remainder of the period of DNA synthesis. After DNA synthesis, the rate of polyadenylated messenger RNA synthesis remained steady for the remainder of the cell cycle.

It is suggested that gene number controls the rates of ribosomal and messenger RNA synthesis. When the number of genes doubles during the DNA synthesis period, the amount of transcription also quickly doubles. Repeat experiments suggested that the genes coding for ribosomal RNA are reproducibly replicated earlier, and over a shorter proportion of the DNA replication period than the genes coding for messenger RNAs. It is calculated that a minimum of 625 genes coding for messenger RNA per haploid genome, transcribed at the maximum rate throughout the cell cycle, would be required to give the observed cell-cycle pattern of messenger RNA synthesis.

The total content of polyadenylated messenger RNA of cells at different stages of the cell cycle was measured by the formation of ribonuclease-resistant complexes between polyadenylic acid sequences in total RNA from non-radioactive yeast and [^{3}H]polyuridylic acid. The amount of polyadenylated messenger RNA per cell rose after the doubling of the rate of polyadenylated messenger RNA synthesis, and by the end of the cell cycle was twice the level at the beginning of the cycle.

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‡ Abbreviations used: G_1, part of interphase preceding DNA synthesis; S, part of interphase during which DNA synthesis occurs; butyl BPD, 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole; oligo(dT), oligodeoxythymidylic acid.

The cell-cycle patterns of messenger and ribosomal RNA synthesis are discussed in relation to the control of balanced growth and doubling of all cellular components during the cell cycle, and in relation to known patterns of enzyme activity during the cell cycle of budding yeast.

1. Introduction

The cell cycle of eukaryotic cells in exponential growth involves the doubling of the DNA and of all other cellular components to produce two daughter cells identical to the parent. The mechanisms by which the duplication of cellular components is co-ordinated to produce this balanced growth are not understood. It is likely, however, that the doubling of gene number during DNA replication may play a part in controlling the doubling of at least some other cellular components. For this reason, it is of interest to examine the pattern of gene activity through the cell cycle by following changes in the rate of RNA synthesis

Previous investigations with eukaryotes have been concerned with either total (Pfeiffer & Tolmach, 1968; Wain & Staatz, 1973), or ribosomal RNA synthesis rates. Control of the rate of rRNA synthesis by the number of rRNA genes present is suggested by some reports (Pfeiffer, 1968; Klevecz & Stubblefield, 1967). Such results provide the basis for a simple model explaining the doubling of the ribosomal population during the cell cycle in exponential growth. However, other reports have described situations where there is no direct link between gene number and the rate of rRNA synthesis (Scharff & Robbins, 1965; Enger & Tobey, 1969; Fraser & Loening, 1974).

The study of changes in activity of the genes coding for proteins has been indirect, by following changes in the activities of enzymes during the cell cycle. However, the recent discovery that many eukaryotic messenger RNAs contain a polyadenylic acid sequence (Darnell *et al.*, 1971; Edmonds *et al.*, 1971; Lee *et al.*, 1971; McLaughlin *et al.*, 1973) and the development of methods for the separation of polyadenylated mRNA from total RNA (Edmonds & Caramela, 1969; Edmonds *et al.*, 1971) have made it possible to study the rate of synthesis of a messenger RNA fraction directly. In this paper we report an investigation of the rates of ribosomal and messenger RNA synthesis during the cell cycle of budding yeast.

In the past, most cell-cycle studies have been made using synchronous cultures. However, many of the techniques used to obtain synchronous cultures from exponentially growing cells may affect the properties of cells during subsequent growth. To avoid the possibility of artifacts introduced by synchronization, we have isolated cells at discrete stages of the cell cycle directly from an exponential culture by zonal centrifugation (Carter *et al.*, 1971; Sebastian *et al.*, 1971). Fractionation of the cell cycle by zonal centrifugation has the further advantages that very large numbers of cells can be handled, and that RNA may be labelled by applying a single pulse of radioactive precursor to an unperturbed exponential culture before fractionation.

2. Materials and Methods

(a) *Yeast and culture conditions*

The yeast used was *Saccharomyces cerevisiae* strain Y185, with genetic markers

$$\frac{\alpha}{a}\ \frac{\text{ade2}}{+}\ \frac{\text{his5}}{+}\ \frac{\text{his8}}{+}.$$

The strain is sucrose negative, which minimizes any possible cellular metabolism during zonal centrifugation on a sucrose gradient. One-litre cultures were grown in flasks at 32°C in yeast extract/peptone/glucose medium (1%:2%:2%) on a rotary incubator at 220 revs/min. The mean generation time was 1·7 h.

For radioactive labelling of nucleic acids, exponential cultures (1×10^7 cells.ml^{-1}) were incubated for 10 min with 16 μCi [2-^{3}H]adenine/ml (Radiochemical Centre, Amersham, U.K.; spec. act. 15·5 Ci.mmol^{-1}). Incorporation was stopped by addition of crushed ice, and the cells were collected by centrifugation at 2000 ***g*** for 5 min at 0°C.

(b) *Cell-cycle fractionation*

Harvested cells (10^{10}) were resuspended in 30 ml yeast extract/peptone/glucose medium at 0°C, and fractionated according to size by zonal centrifugation (Carter *et al.*, 1971; Halvorson *et al.*, 1971) on a 15% to 40% sucrose gradient in the M.S.E. type A zonal rotor, pre-cooled to 4°C. The rotor was run at 600 revs/min during loading and unloading and at 2000 revs/min to separate the cells. Unloading was begun after about 10 min at 2000 revs/min when the largest cells had migrated about $\frac{2}{3}$ of the distance to the edge of the rotor. 40% sucrose solution was pumped into the outer edge of the rotor to displace the gradient towards the centre and 10-ml fractions of the gradient were collected. The cells in each fraction were sedimented at 2000 ***g*** for 5 min at 0°C, then resuspended in 5 ml ice-cold extraction medium (2% sodium triisopropylnaphthalene sulphonate, 50 mM-Tris·HCl (pH 7·8); 10 mM-NaCl). 0·1 ml of the suspension was diluted with 0·9% NaCl, and cell number, volume distribution and mean cell volume were determined in the Coulter Electronic cell counter. Fractionation on the zonal rotor and resuspension in extraction medium did not affect cell number or mean cell volume.

The percentage of cells with buds was found by microscopic examination of 200 cells from fractions from the zonal rotor.

Synchronous cultures were prepared from exponential cultures by the method of Mitchison & Vincent (1965). Mean cell volume was determined on samples taken at intervals during the cell cycle.

(c) *Measurement of [^{3}H]adenine uptake*

Three 50-μl samples of the suspension of yeast cells in extraction medium were dried on 20-mm squares of Whatman GF/A glass fibre paper, and counted in 5 ml 0·5% butyl PBD/toluene scintillator in a Packard liquid scintillation counter. The remainder of the cell suspension was frozen and stored at −20°C.

(d) *Extraction of nucleic acids*

The frozen yeast suspension was thawed at 0°C. 2 g of glass beads (40 mesh) were added and the cells were broken in a Vibromixer (Shandon Scientific, London, U.K.) by 5 min agitation at maximum setting. The temperature of the homogenate was brought to 20°C. An equal volume of phenol mixture (phenol, 1000 g; redistilled *m*-cresol, 200 ml; 8-hydroxyquinoline, 1 g; chloroform, 300 ml; water, 200 ml) was added and the mixture was shaken for 5 min. The aqueous and organic phases were separated by centrifugation at 2000 ***g*** for 5 min. The organic phase was re-extracted with a further 2 ml of extraction medium, then the 2 aqueous phases were combined and extracted twice more with equal volumes of phenol mixture lacking chloroform. Nucleic acids were precipitated from the final aqueous phase by addition of NaCl to 0·3 M and 2·5 vol. ethanol, and storage for 12 h at 0°C.

The precipitated nucleic acids were sedimented at 2000 ***g*** for 5 min. Three 20-μl samples of the supernatant were dried on 20-mm squares of Whatman GF/A and counted in butyl PBD/toluene scintillator as above, to give an estimate of the radioactivity of the alcohol-soluble pool.

The nucleic acids were further purified by dissolving in 4 ml 0·5% sodium dodecyl sulphate, 0·15 M-sodium acetate (pH 6·0) and reprecipitating with 2·5 vol. ethanol at

0°C for 12 h. After 3 such cycles of reprecipitation, the nucleic acids were stored as a suspension in 80% ethanol, 0·1% sodium dodecyl sulphate, 0·03 M-sodium acetate (pH 6·0), at 2°C.

(e) *Determination of yield of RNA*

A portion of the nucleic acid suspension was sedimented and hydrolysed in 0·5 M-$HClO_4$ at 70°C for 20 min. The ultraviolet absorption spectrum of the hydrolysate was measured in the Unicam SP800 recording spectrophotometer. The RNA concentration of the extract was calculated using a molar absorption coefficient of 12,400 $M^{-1}.cm^{-1}$, calculated for a solution of equal molar quantities of the 4 ribonucleotides at acid pH. DNA, which was also present in the extract, is only 1% of total nucleic acid in *S. cerevisiae*, and thus did not interfere significantly with this assay of total RNA content. Control experiments showed that the yield of RNA by the Vibromixer–phenol extraction method was around 90% of the total RNA content of the yeast.

(f) *Polyacrylamide gel electrophoresis*

Nucleic acids were fractionated on 6-mm diameter, 70-mm long polyacrylamide gels as described by Loening (1967). The gels had final concentrations equivalent to 2·4% acrylamide and 0·12% bis-acrylamide. Electrophoresis was for 2·5 h at 5 mA per gel, 8 V per cm gel length. The gels were scanned for absorbance at 265 nm in a Joyce-Loebl gel scanner, frozen in solid CO_2, then cut transversely into 0·5-mm slices. Each slice was incubated with 0·3 ml 0·5 M-$HClO_4$ for 20 min at 70°C. 7 ml scintillator (0·5% butyl PBD; 40% 2-methoxyethanol; 60% toluene) were added and radioactivity was determined by scintillation counting.

(g) *Determination of DNA per cell and DNA specific activity*

Between 50 and 150 μg extracted total nucleic acid was dissolved in 0·1 ml 0·15 M-NaCl, 0·015 M-sodium citrate (pH 6·0), and digested with 10 μg pancreatic ribonuclease/ml (EC 2.7.7.16) at 0°C for 10 min. Sodium dodecyl sulphate was added to 0·2%, and the samples were fractionated by polyacrylamide gel electrophoresis. Figure 1 shows the ultraviolet absorption and radioactivity profile of a typical gel. DNA was detectable as a sharp peak near the top of the gel; the broken-down fragments of RNA migrated much more rapidly.

The weight of DNA on the gel was calculated from the peak area of the DNA ultraviolet absorption peak. Peak area is linearly proportional to the weight of nucleic acid in the peak (Fraser, 1971). The total radioactivity of the DNA was found by adding the radioactivities of the gel slices making up the DNA radioactivity peak. The specific activity of the DNA was calculated by dividing radioactivity by the ultraviolet absorption peak area.

Double-stranded viral RNA (Bevan *et al.*, 1973) is found in this strain of yeast, though the concentration varies widely. This double-stranded RNA migrated only slightly faster than DNA on gels, but did not interfere with the assay of DNA amount and radioactivity, as it is degraded by the RNase treatment. Control experiments also showed that the DNA absorption and radioactivity peaks were eliminated by treatment of the sample with 10 μg DNase/ml (Sigma, RNase-free) for 10 min at 0°C, in 50 mM-2-(*N*-morpholino) ethanesulphonic acid (pH 7·0) and 2 mM-magnesium acetate.

(h) *Determination of specific activity of ribosomal RNA*

Between 10 and 30 μg total nucleic acid were fractionated on polyacrylamide gels. Figure 2(a) shows a typical ultraviolet absorption and radioactivity profile. The gel shows labelling of polydisperse RNA and also 5 distinct peaks. These include the mature rRNAs with molecular weights $1{\cdot}3\times10^6$ and $0{\cdot}7\times10^6$, and 3 rRNA precursor species with molecular weights of $2{\cdot}5\times10^6$, $1{\cdot}6\times10^6$ and $0{\cdot}8\times10^6$ (Udem & Warner, 1972). The total radioactivity in each peak was determined from the sum of the radioactivities in the constituent gel slices, after subtracting the background of polydisperse radioactivity, as shown in Figure 2(a). Delimitation of the radioactivity peaks from the background was done without knowledge of sample number, to avoid introduction of subjective bias.

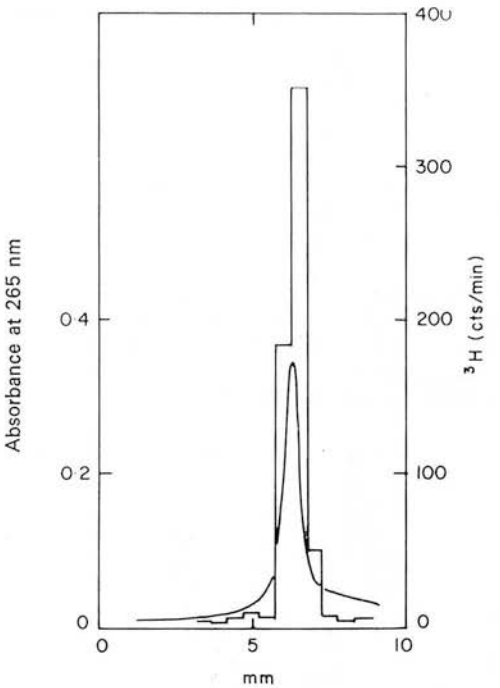

FIG. 1. Polyacrylamide gel electrophoresis of [2-^{3}H]adenine-labelled yeast DNA. The continuous line shows absorbance at 265 nm; the histogram shows radioactivity. An exponentially growing yeast culture was labelled for 10 min with [^{3}H]adenine, then fractionated on the zonal rotor. 100 μg total nucleic acid from a fraction near the middle of the cell cycle was digested with RNase prior to electrophoresis.

The specific activity of the rRNA was calculated by dividing the total radioactivity of the 3 precursor and 2 mature rRNA peaks by the combined peak areas of the $1{\cdot}3\times10^6$ and $0{\cdot}7\times10^6$ dalton ultraviolet absorption peaks. The very small amount of ultraviolet absorption of the $2{\cdot}5\times10^6$ dalton precursor, which migrates with the DNA, was neglected. The radioactivity in the DNA was so low in comparison to rRNA radioactivity that it did not significantly affect the rRNA radioactivity measurement.

As a test of the reproducibility of measurement of specific activity of rRNA by this method, a radioactive yeast culture was split into 8 parts. Nucleic acids were extracted from each and specific activity of rRNA was measured by gel electrophoresis. The standard deviation of the 8 values was 4% of the mean value, indicating that reproducibility is high.

(i) *Preparation of polyadenylated mRNA by oligo(dT)-cellulose*

Between 20 and 100 μg total nucleic acid were dissolved in 1 ml binding buffer (NaCl, 400 mM; EDTA, 1 mM; Tris·HCl, 10 mM, pH 7·8; sodium dodecyl sulphate 0·2%) and shaken gently for 30 to 60 min at 20°C with 20 mg oligo(dT)-cellulose (Collaborative Research Inc., Waltham, Mass, U.S.A.; binding capacity 42·8 A_{260} units poly(A).g^{-1}). The cellulose was sedimented at 12,000 *g* for 1 min, then washed 4 times with 1·0 ml binding buffer. Those RNAs lacking a polyadenylic acid sequence (poly(A)$^-$RNA) were precipitated from the combined binding buffer supernatants by addition of 2·5 vol. ethanol and storage for 12 h at 0°C. Figure 2(b) shows a polyacrylamide gel fractionation of a typical poly(A)$^-$RNA fraction.

The cellulose was then washed 4 times with 0·5 ml elution buffer (binding buffer minus NaCl) to release the poly(A)$^+$RNA (RNA containing a polyadenylic acid sequence). The elution buffer washes were combined. Where the poly(A)$^+$RNA was to be analysed by gel electrophoresis, 50 μg non-radioactive yeast carrier RNA were added and RNA was

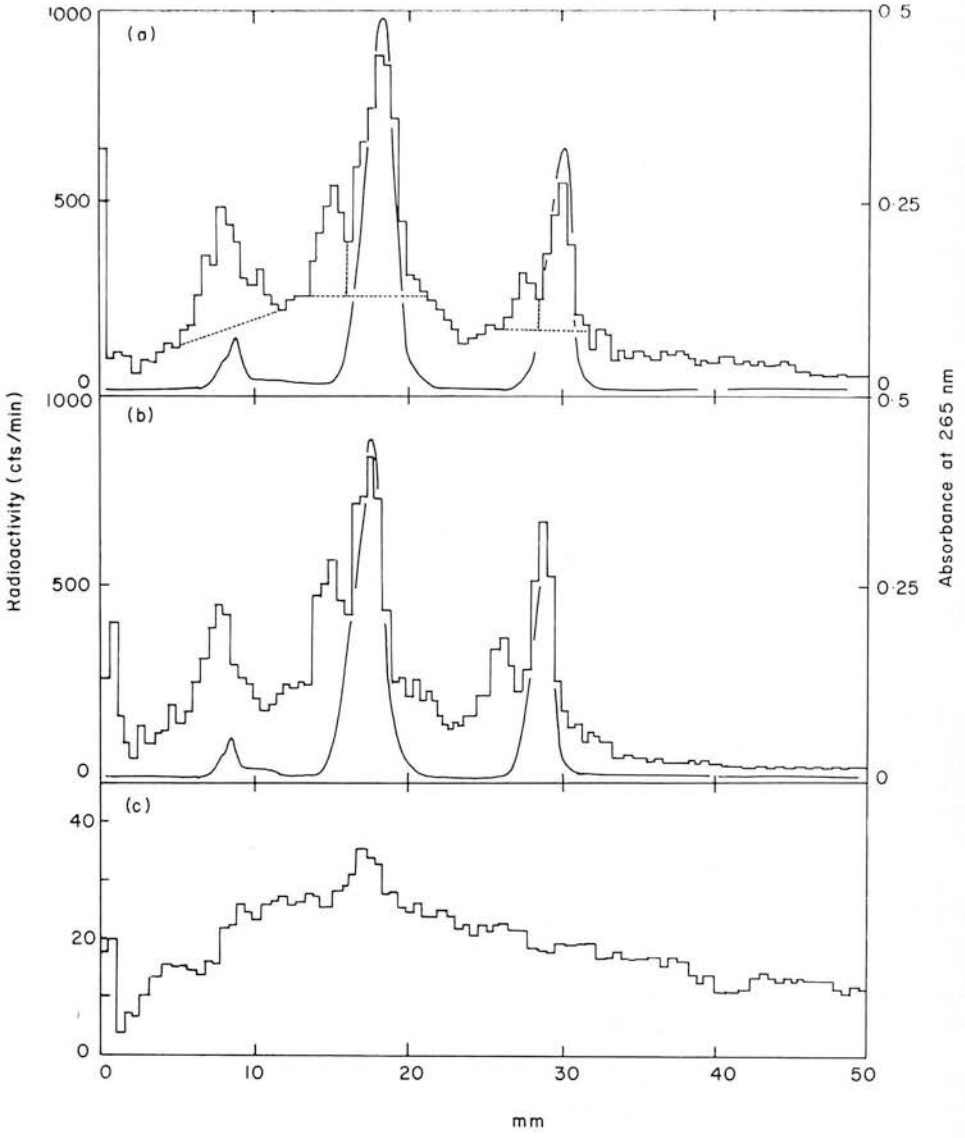

FIG. 2. Polyacrylamide gel electrophoresis of yeast nucleic acid fractions. An exponential culture was labelled for 10 min with [^{3}H]adenine, then fractionated by zonal centrifugation. The Figure shows nucleic acids from a fraction near the middle of the cell cycle. The continuous lines show absorbance at 265 nm; the histograms show radioactivity. (a) Total nucleic acid, showing rRNA radioactivity peaks at 8, 15, 18, 27 and 30 mm, corresponding to molecular weights of $2{\cdot}5\times10^6$, $1{\cdot}6\times10^6$, $1{\cdot}3\times10^6$, $0{\cdot}8\times10^6$ and $0{\cdot}7\times10^6$. The broken lines show the delimitation of rRNA radioactivity peaks from the polydisperse background radioactivity, used in the calculation of rRNA specific activity. (b) Non-polyadenylated nucleic acids and (c) polyadenylated RNA, prepared by fractionation on oligo(dT)-cellulose. Radioactivity values on these scans are expressed per 100 μg total RNA.

precipitated by addition of NaCl to 0·3 M and 2·5 vol. ethanol, and stored at -20°C for 12 h. Figure 2(c) shows a gel separation of a typical poly(A)$^+$RNA fraction.

To determine the total radioactivity of the poly(A)$^+$RNA, the combined elution buffer washes were dried in scintillation vials at 70°C, then hydrolysed in 0·3 ml 0·5 M-$HClO_4$ at 70°C for 20 min. [^{3}H]radioactivity was determined by scintillation counting after

addition of 7 ml butyl PBD/2-methoxyethanol/toluene scintillator. A "specific activity" for the poly(A)$^+$RNA was found by dividing its total radioactivity by the weight of RNA fractionated on oligo(dT)-cellulose. The actual specific activity of poly(A)$^+$RNA (radioactivity per unit weight poly(A)$^+$RNA) could not be determined, as the weight of poly(A)$^+$RNA prepared by oligo(dT)-cellulose fractionation was too low to measure.

(j) *Measurement of total polyadenylated RNA content*

The amount of polyadenylated RNA per unit total RNA was estimated by measuring the formation of RNase-resistant complexes between the poly(A) sequences in non-radioactive total RNA and [^{3}H]polyuridylic acid (Bishop *et al.*, 1974). Non-radioactive yeast cells from an exponential culture were fractionated on the zonal rotor as above, and RNA was extracted from each fraction. 20-μg samples of RNA were dissolved in 0·2 ml 50 mM-Tris·HCl (pH 7·6); 150 mM-NaCl; 0·5% sodium dodecyl sulphate. 0·1 to 0·2 μg [^{3}H]polyuridylic acid (Miles Laboratories Inc., spec. act. 0·2 μCi.μg^{-1}; molecular weight 50,000), dissolved in the same buffer at 0·01 μg.ml^{-1}, was added. The amount of [^{3}H]poly(U) added was 5 to 10 times the amount which formed RNase resistant complexes with the RNA. After 30 min at 25°C, 50 μl 1 M-KCl was added to precipitate the sodium dodecyl sulphate. The mixtures were cooled to 0°C and digested with 20 μg pancreatic RNase/ml for 20 min. 1 ml 0·5% sodium dodecyl sulphate, 0·15 M-sodium acetate (pH 6·0), containing 100 μg non-radioactive yeast carrier RNA was added, and RNase-resistant complexes were precipitated by addition of 3 ml ethanol, and stored for 3 h at −20°C. The precipitates were collected by filtration on Whatman GF/A glass fibre paper, and washed 3 times with 5 ml 80% ethanol containing 0·1 M-NaCl and 100 μg uridine/ml. Alcohol-insoluble radioactivity on the filters was determined after drying by counting in butyl PBD/toluene scintillator. The poly(A)$^+$RNA content was expressed as alcohol-insoluble radioactivity per unit weight of total RNA.

Control experiments showed that when the RNase digestion was omitted, 95 to 100% of the input of [^{3}H]poly(U) radioactivity was recovered. When [^{3}H]poly(U) was mixed with poly(A)$^-$RNA prepared by oligo(dT)-cellulose fractionation as above, between 0 and 2% of the input radioactivity remained alcohol-precipitable after RNase treatment. Fractionation of the complexes formed between [^{3}H]poly(U) and total RNA by sedimentation on sucrose gradients (Fraser & Loening, 1973) showed a polydisperse distribution of radioactivity, from 6 S to over 30 S. Free poly(U) sedimented alone had a sedimentation coefficient of 3 S. This confirms that the [^{3}H]poly(U) was complexed to high molecular weight RNAs, with a range of sizes such as would be expected for mRNA.

(k) *Calculation of the rates of rRNA and poly(A)$^+$RNA synthesis per cell, and poly(A)$^+$RNA content per cell*

For each zonal rotor fraction of yeast, the specific activities of rRNA or poly(A)$^+$RNA determined as above were multiplied by the value for RNA per cell for that fraction, to give rates of rRNA and poly(A)$^+$RNA synthesis per cell. The amounts of poly(A)$^+$RNA and DNA per unit total RNA were similarly treated to give weights of poly(A)$^+$RNA and DNA per cell. The values for RNA per cell for fractions across the zonal rotor were taken from the line best fitting all points, rather than using the measured experimental value for each fraction. This served to reduce variation arising from small differences in the efficiency of extraction of RNA from different fractions (Fig. 6).

3. Results

(a) *Cell-cycle analysis*

Yeast cells increase in size during the cell cycle, and can be separated according to size by zonal centrifugation. Thus the zonal rotor may be used to separate cells into fractions representing different stages of the cell cycle (Carter *et al.*, 1971; Sebastian *et al.*, 1971). Figure 3 shows a fractionation on the zonal rotor of cells from an exponential culture of *S. cerevisiae*. It is clear that there has been a fractionation according

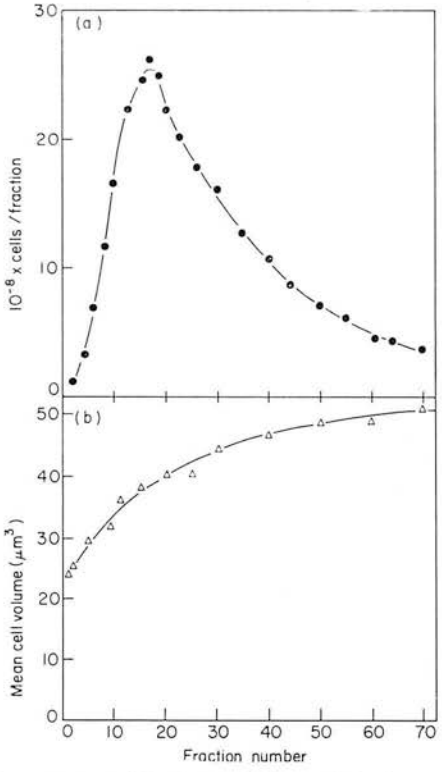

FIG. 3. Fractionation of an exponential culture of *S. cerevisiae* by zonal centrifugation. (a) Cells per 10-ml fraction of gradient from the zonal rotor. (b) Mean cell volume in fractions of the gradient.

to cell size, and that mean cell volume doubles over the region of the gradient containing the cells.

The variation in number of cells per fraction across the gradient (Fig. 3(a)) is a consequence of two factors: (1) the frequency of cells of different age classes, hence different sizes, in an exponentially growing culture, and (2) the geometry of the zonal rotor, which causes progressive dilution of cells with distance from the rotor centre. Sebastian *et al.* (1971) have shown that the type of distribution of cell number per fraction shown in Figure 3(a) agrees with that calculated from the distribution of cell ages in an exponential culture and the rotor geometry.

Cells in individual fractions can be attributed to a particular phase of the cell cycle if the sizes of cells at different times during the cell cycle are known. Figure 4 shows that in a synchronous culture under the same growth conditions, mean cell volume increased linearly, and doubled during the cell cycle. The range of cell volumes during the cell cycle in synchronous culture (Fig. 4) was similar to the range of sizes fractionated on the zonal rotor. Accordingly, we have been able to use cell volume as a cell-cycle marker, to correlate fractions from the zonal rotor with their stage in the cell cycle.

To determine the pattern of nucleic acid synthesis during the cell cycle, we labelled exponentially growing yeast cultures with [^{3}H]adenine for 10 minutes, i.e. about 0·1 of a cell cycle. In asynchronous, exponential cultures, there are cells at every stage of the cell cycle. When the culture is harvested and separated by zonal centrifugation,

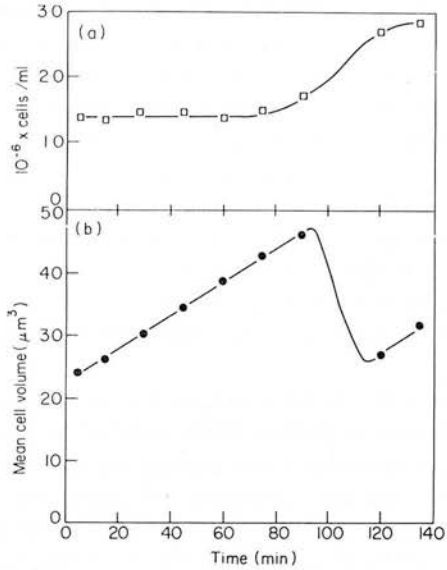

FIG. 4. Changes in (a) cell number per ml and (b) mean cell volume with time in a synchronous culture of *S. cerevisiae*. The synchronous culture was prepared by selecting small cells near the beginning of the cell cycle from the top of a sucrose gradient fractionation of an exponential culture (Mitchison & Vincent, 1965).

a series of fractions is obtained representing cells at different stages of the cell cycle which had been pulse-labelled with [^{3}H]adenine for the previous 0·1 of the cycle.

(b) *Characterization of the RNA fractions examined through the cell cycle*

Figure 2(a) shows polyacrylamide gel electrophoresis of total RNA which was extracted from one fraction of yeast from a zonal rotor separation. The radioactivity profile shows a background of polydisperse RNA, and five peaks; the $1{\cdot}3\times10^6$ and $0{\cdot}7\times10^6$ dalton mature rRNAs, and the $2{\cdot}5\times10^6$, $1{\cdot}6\times10^6$ and $0{\cdot}8\times10^6$ dalton rRNA precursors (Retel & Planta, 1967; Udem & Warner, 1972). After a 10-minute pulse with [^{3}H]adenine, about half of the total radioactivity in rRNA was in the precursor forms.

Figure 2(c) shows a gel of poly(A)$^+$RNA prepared from total RNA by fractionation on oligo(dT)-cellulose. The radioactivity was polydisperse on the gel. Using the approximately linear relationship between log molecular weight and electrophoretic mobility (Loening, 1969), and taking the $1{\cdot}3\times10^6$ and $0{\cdot}7\times10^6$ dalton rRNAs as standards, the polydisperse poly(A)$^+$RNA covers a molecular weight range from 3×10^6 down to less than $2{\cdot}5\times10^5$. On sucrose gradients the polydisperse poly(A)$^+$RNA sediments in the range from 6 S to 35 S. The poly(A)$^+$RNA was between 10 and 15% of total acid-insoluble radioactivity.

The poly(A)$^+$RNA fraction showed several features characteristic of mRNA: it was heterogeneous in size, was labelled to a high specific activity during a pulse incubation, and contained a poly(A) sequence. We have studied the poly(A)$^+$RNA as a model for messenger RNA. However, the poly(A)$^+$RNA probably does not represent the total mRNA content of the cell. Eukaryotic mRNAs lacking a poly(A) sequence have been found (Adesnik & Darnell, 1972; Milcarek *et al.*, 1974; Greenberg, 1975). Figure 2(b) shows that some polydisperse RNA was also present in the RNA fraction

which failed to bind to oligo(dT)cellulose at high salt concentration. It is difficult to estimate the amount of this RNA, but it may account for up to half of the total polydisperse RNA. At least some of this poly(A)$^-$ polydisperse RNA might have messenger activity. McLaughlin *et al.* (1973) found in yeast that some of the polyribosome-associated polydisperse RNA did not contain a poly(A) sequence. Shiokawa & Pogo (1974), however, found the poly(A)$^-$ polydisperse RNA of yeast mainly in the nuclear and membrane fractions.

We conclude that the poly(A)$^+$RNA fraction is likely to represent a major portion, though probably not all, of the cellular mRNA. The possibility cannot be excluded that some of the poly(A)$^+$RNA is not mRNA, though no functions for poly(A)$^+$ RNA other than those of mRNA or presumed mRNA precursors have yet been reported.

Fractionation of yeast cells on the zonal rotor took approximately 40 minutes. It was important to check that no further RNA metabolism was occurring during this time. Accordingly, an exponential yeast culture was labelled for 10 minutes with [^{3}H]adenine, divided into two parts and each half chilled by addition of crushed ice. The cells from one portion were immediately collected, resuspended in extraction medium and frozen at −20°C. The cells from the remaining portion were collected by centrifugation and resuspended in 27·5% sucrose at 4°C for one hour, to simulate fractionation on the zonal rotor. The cells were then collected by centrifugation, resuspended in extraction medium and frozen. RNA was prepared from both samples. There were no differences between the samples in total radioactivity of the RNA, pattern of radioactivity after fractionation on polyacrylamide gels, percentage of total rRNA radioactivity in the rRNA precursor molecules, percentage of total radioactivity in the poly(A)$^+$RNA, or distribution of the poly(A)$^+$RNA after electrophoresis. These results confirm that no further synthesis, processing or degradation of RNA occurred during zonal fractionation of the cells.

(c) *DNA synthesis through the cell cycle*

In yeasts, it is not possible to study DNA synthesis by labelling specifically with [^{3}H]thymidine, as the cells do not contain thymidine kinase (Brendel & Haynes, 1972). The approach we have adopted is to label both DNA and RNA with [^{3}H]-adenine, degrade the RNA by RNase treatment, and then separate the DNA peak from the degradation fragments of RNA by polyacrylamide gel electrophoresis (Fig. 1). Figure 5 shows changes in the specific activity of the DNA after a 10-minute pulse of [^{3}H]adenine, and changes in DNA content per cell, during the cell cycle. It is clear from the specific activity data that the major period of DNA synthesis was from 0·4 to 0·8 of the cycle as fractionated on the rotor. DNA content per cell began to rise at 0·4 of the cycle, and had approximately doubled by 0·8 of the cycle.

In budding yeast, the start of DNA synthesis coincides with the time of bud emergence (Williamson & Scopes, 1962; Williamson, 1965). Figure 5 shows that in cells fractionated on the zonal rotor, bud emergence also coincided with the onset of DNA synthesis.

Our estimate of the time of occurrence of DNA synthesis (S phase) in the cell cycle varies from previous reports. In an autoradiographic study, Williamson (1965) found that S phase occupied the first 27% of the budded phase of the cell cycle; our results show S phase occupying a larger proportion of the budded phase. This is a conse-

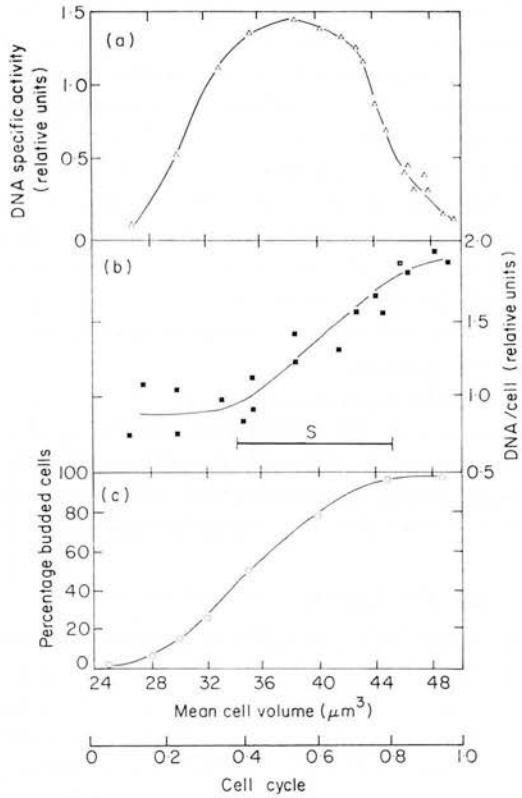

FIG. 5. DNA synthesis during the cell cycle of *S. cerevisiae*. An exponentially growing culture, labelled for 10 min with [2-^{3}H]adenine, was fractionated according to cell volume on the zonal rotor. The specific activity (a) and amount of DNA per cell (b) were determined by polyacrylamide gel electrophoresis. The lower scale shows the correlation of mean cell volume with position in the cell cycle, from Fig. 4. The horizontal bar shows the position of the period of DNA synthesis; this is reproduced in Figs 7 and 8 showing changes in rates of RNA synthesis. (c) Shows changes in the percentage of budding cells across the zonal rotor.

quence of the imperfect fractionation of cells on the zonal rotor, and is analogous to the situation in synchronous cultures, where S phase in the culture is longer than S in the individual cell because of imperfections in synchrony (Williamson, 1965).

S phase in the zonal rotor fractionated cell cycle also started much later than in previous studies using autoradiography (Williamson, 1965) or synchronous cultures (Williamson & Scopes, 1962; Williamson, 1965). Part of this difference was due to the longer duration of the unbudded (G_1) phase in our cells. Williamson (1965) found 8 to 10% unbudded cells in exponential cultures, whereas in undisturbed exponential cultures of our strain of yeast 16% of cells were unbudded.

A second cause of the extended G_1 phase was a feature of fractionation of the cell cycle using the zonal rotor. Cells at the beginning of the cycle are not all the same size, but are normally distributed about a mean. The first few fractions from the zonal rotor probably contain cells with a mean volume less than the mean volume of all cells at the start of the cycle. Theoretically, the start of the cycle should be taken as that fraction where mean cell volume equals the mean cell volume of all cells at the start of the cycle. Identification of this fraction is difficult. Synchronous cultures

cannot be used to find the mean cell volume at the start of the cycle, as the conditions of synchronization and growth of synchronous cultures may themselves introduce differences in cell size from unperturbed, exponentially growing cells, and as the least imperfection in synchrony will make it difficult to measure only cells at the beginning of the cycle. We have therefore decided to include all cells at the top of the gradient in the cell cycle. This has the consequence of lengthening the G_1 phase. To put this artifact of the zonal fractionation into perspective, it should be emphasized that the first 20% of the cycle contains only about 1% of all cells on the rotor. The difficulty of fixing the start (and similarly, the end) of the cell cycle means that the zonal rotor approach is less suitable for determining the absolute timing of events in the cell cycle, than for determining the temporal relationship between two events such as DNA synthesis and RNA synthesis.

A third factor which might result in a comparatively long G_1 period is that during harvesting and fractionation of cells, premature separation of buds would give rise to excessive numbers of small cells. This did not occur to any significant extent in our fractionation: the overall percentage of unbudded cells after zonal rotor fractionation was 20 to 22, little higher than the 16% unbudded cells in undisturbed exponential cultures.

(d) *RNA synthesis through the cell cycle*

Figure 6 shows that total RNA per cell rose continuously through the cell cycle, and approximately doubled during the cycle. The rise was close to exponential (the value of r for the linear regression of log RNA on mean cell volume was 0·96; $n = 33$). However, possible slight variations in the rate of RNA accumulation would be masked by variations in the efficiency of extraction of RNA from different fractions.

Figure 7 shows changes in the rate of synthesis of rRNA during the cell cycle, calculated from polyacrylamide gel fractionations such as those shown in Figure 2.

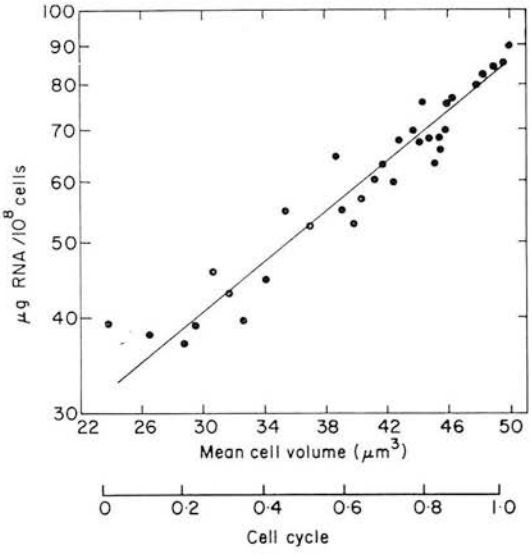

FIG. 6. Changes in RNA content of yeast cells during the cell cycle. Cells from an exponentially growing culture were fractionated by volume on the zonal rotor. A straight line was fitted to the data by the method of least squares for the regression of log RNA on mean cell volume. The value of r is 0·96; $n = 33$.

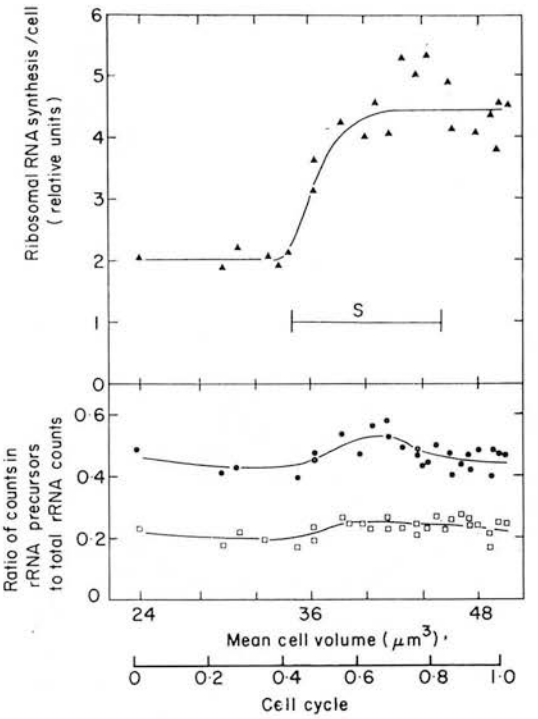

FIG. 7. Changes in the rate of ribosomal RNA synthesis (a) and in the rate of processing of ribosomal RNA precursors to mature rRNAs (b) during the cell cycle. An exponential culture was labelled with [2-^{3}H]adenine for 10 min, then fractionated according to cell volume on the zonal rotor. The total radioactivity incorporated into rRNA (precursors and mature forms) was determined by gel electrophoresis (—▲—▲—). The rate of processing of precursors to mature rRNAs was expressed as (1) the ratio of radioactivity in the $2{\cdot}5\times10^6$ dalton precursor to total (precursor plus mature) rRNA radioactivity (—□—□—) or (2) the ratio of radioactivity in the three rRNA precursor molecules ($2{\cdot}5\times10^6$, $1{\cdot}6\times10^6$ and $0{\cdot}8\times10^6$ daltons) to total rRNA radioactivity (—●—●—). The horizontal bar in (a) shows the timing of DNA synthesis, from Fig. 5.

In the experiment shown, the rate of rRNA synthesis remained constant for the first 0·4 of the cycle. Between 0·4 and 0·6 there was a sharp doubling in the rate of rRNA synthesis per cell, which then remained constant for the remainder of the cycle. The time of the doubling in the rate of rRNA synthesis was early in S phase.

The rate of processing of precursor rRNA to mature rRNA was examined by the ratio of radioactivities in (a) $2{\cdot}5\times10^6$ dalton rRNA precursor; and (b) the $2{\cdot}5\times10^6$, $1{\cdot}6\times10^6$ and $0{\cdot}8\times10^6$ dalton precursors, to the total radioactivity in all five rRNA species. Both ratios show that after the increase in the rate of rRNA synthesis, a higher percentage of the rRNA labelling was in the precursor forms than before the increase in synthesis (Fig. 7). Later in the cycle, the percentage of labelled rRNA in the precursor forms fell back to the level pertaining at the start of the cycle.

Poly(A)$^+$RNA synthesis per cell remained constant for the first 0·5 of the cell cycle, then increased between 0·5 and 0·85 (Fig. 8). The increase was slightly more than a doubling. The rate of poly(A)$^+$RNA synthesis then remained constant for the remaining part of the cycle. The time of the increase in rate of poly(A)$^+$RNA synthesis coincided with the later part of S phase. This step in synthetic rate was thus considerably later than the step in rate of rRNA synthesis (Fig. 7). Also, the step in the

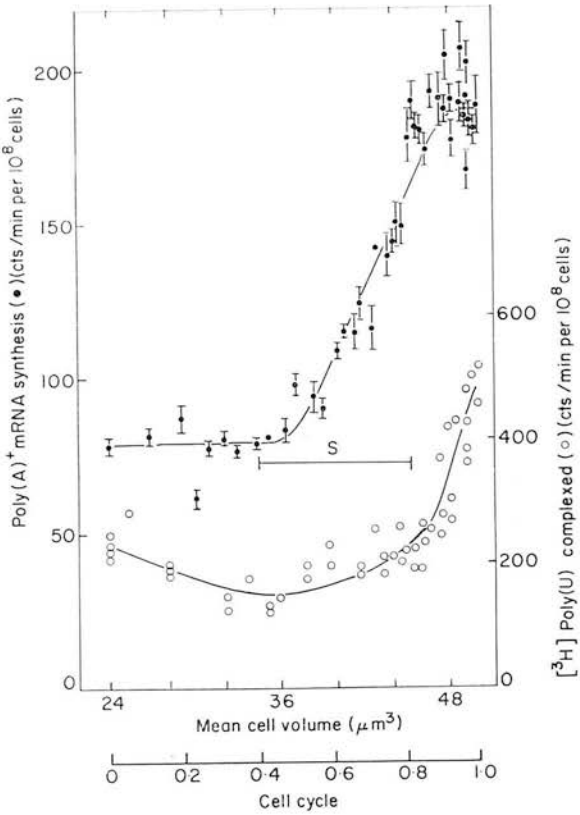

FIG. 8. Changes in the rate of synthesis of polyadenylated RNA (●) and in the content per cell of polyadenylated RNA (○) during the cell cycle of *S. cerevisiae*. For measurement of poly(A)$^+$ RNA synthesis, an exponential culture was pulse-labelled for 10 min with [2-^{3}H]adenine, then fractionated according to cell volume on the zonal rotor. The horizontal bar shows the time of DNA synthesis, from Fig. 5. The vertical bars show the standard errors of the means. Each point is the mean of between 4 and 8 determinations. For measurement of poly(A)$^+$ RNA content, non-radioactive exponential phase yeast cells were fractionated on the zonal rotor. Poly(A)$^+$RNA content was measured by the formation of RNase-resistant complexes between total RNA and [^{3}H]poly(U).

rate of poly(A)$^+$RNA synthesis occupied a much larger proportion of the cell cycle than the comparatively sharp step in rate of rRNA synthesis. Figure 9 shows a cell cycle map on which are plotted the times of increases in the rates of poly(A)$^+$RNA and rRNA synthesis, in three replicate experiments of the type shown in Figures 7 and 8. It is clear that in all experiments, the increase in the rate of rRNA synthesis was earlier than the increase in rate of poly(A)$^+$RNA synthesis, and that the step in rRNA synthetic rate consistently occupied a smaller proportion of the cell cycle than the step in rate of poly(A)$^+$RNA synthesis.

A danger when measuring rates of RNA synthesis by incorporation of radioactive precursor is that any change in the rate of incorporation may be a consequence of a change in the rate of uptake of the precursor, or of the specific activity of the precursor pool, rather than a change in the actual rate of synthesis of RNA. However, two aspects of our results suggest that changes in precursor uptake or pool specific

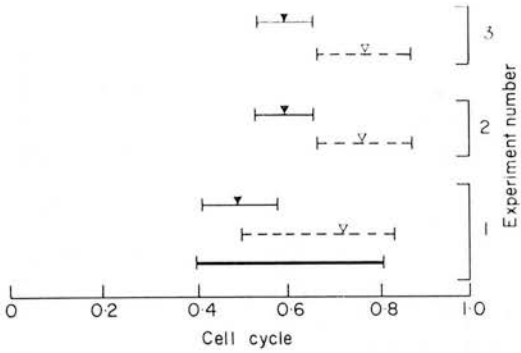

FIG. 9. Map of the cell cycle of *S. cerevisiae* showing times of changes in rates of nucleic acid synthesis. The duration of the rise in the rate of synthesis of rRNA (|————————|) and poly(A)$^+$-RNA (|– – – – – –|), and the times when the rates of synthesis were half-way between the pre-increase and post-increase levels ((▼) rRNA; (▽) poly(A)$^+$RNA)) are shown for 3 separate experiments of the type shown in Figs 7 and 8. The timing of DNA synthesis was determined for one of the experiments and is shown (————————).

activity were not responsible for changes in the rate of adenine incorporation into rRNA and poly(A)$^+$RNA. Firstly, the total uptake of adenine per cell, and the radioactivity of the alcohol-soluble pool both increased continuously through the cell cycle, with no suggestion of a step (Fig. 10). Secondly, the times of the steps in rates of incorporation of adenine into rRNA and poly(A)$^+$RNA were consistently different. If the steps were due to changes in precursor uptake rate or specific activity, they would be expected to occur simultaneously. We conclude that the changes in the rates of incorporation of [^{3}H]adenine into rRNA and poly(A)$^+$RNA represent changes in the rates of synthesis of these molecules.

In addition to measuring the rate of poly(A)$^+$RNA synthesis at different stages of the cell cycle, we have measured changes in the total polyadenylated RNA content of cells, by assaying the amount of [^{3}H]poly(U) which formed RNase-resistant complexes with poly(A) sequences in total RNA. As shown in Figure 8, the amount of poly(A) per cell declined for the first 0·4 of the cell cycle. At 0·5, which also marked the start of the increase in rate of poly(A)$^+$RNA synthesis, the poly(A)$^+$RN content per cell began to increase slowly. Very late in the cycle, there was a major accumulation of poly(A)$^+$RNA, which by the end of the cycle had reached twice the value at the beginning of the cycle.

4. Discussion

(a) *RNA synthesis during the cell cycle*

The rates of poly(A)$^+$RNA (Fig. 8) and rRNA (Fig. 7) synthesis were constant during G_1, doubled during S phase, then again became constant at the new level for the remainder of the cell cycle. There was no evidence of any drop in the rate of RNA synthesis during nuclear division, at about 0·8 of the cycle. In higher eukaryotes, there is a decline in the rate of RNA synthesis during mitosis (Das, 1963; Scharff & Robbins, 1965) caused by the unavailability of the contracted mitotic chromosomes for transcription.

A likely explanation of the increases in poly(A)$^+$RNA and rRNA synthesis rates

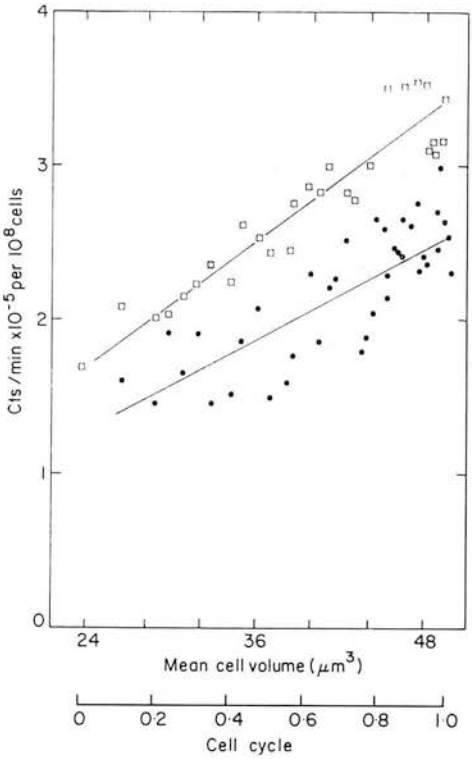

FIG. 10. Changes in the uptake of [2-^{3}H]adenine (□) and in the total radioactivity of the alcohol-soluble pool (●) during the cell cycle of *S. cerevisiae*. An exponential culture, labelled for 10 min with [2-^{3}H]adenine, was fractionated according to cell volume by zonal centrifugation.

during S phase is that the number of genes is the factor controlling the rate of transcription. The simplest form of gene dosage mechanism predicts that the increase in synthetic rate should occur during S phase, and that the rate increase should be a doubling. Our results for rRNA and poly(A)$^+$RNA synthesis rates are in agreement with both predictions. But although our results strongly suggest that gene dosage was the factor controlling the amount of transcription, they do not completely prove a casual relationship between DNA replication and increases in the rate of RNA synthesis. A test of this causality requires experiments in which DNA synthesis is inhibited, or S phase is shifted to a different part of the cycle, and the effects of these treatments on RNA synthesis are examined. These experiments will be reported separately.

Other workers have reported a doubling in the rate of total (Pfeiffer & Tolmach, 1968; Crippa, 1966) and rRNA (Pfeiffer, 1968; Klevecz & Stubblefield, 1967) synthesis during S phase in higher eukaryotes, and have suggested that gene dosage is responsible. To our knowledge, a gene dosage effect on the rate of synthesis of a major, defined fraction of mRNA has not previously been reported. Pfeiffer (1968) did find a doubling during S phase in HeLa cells of the rate of synthesis of a pulse-labelled, cytoplasmic RNA fraction, of which mRNA was probably the major component. In an investigation of the rate of poly(A)$^+$RNA synthesis in selection synchronized cultures of the fission yeast *Schizosaccharomyces pombe*, we have also found a doubling in the synthetic rate during the period of DNA replication (Fraser & Moreno, 1976).

In contrast to our suggestion that the rate of rRNA synthesis doubles during S phase, Sogin *et al.* (1974) found that the rate of rRNA synthesis in budding yeast increases exponentially through the cell cycle. The explanation of this difference probably lies in the different culture conditions used. Sogin *et al.* (1974) grew their yeast on a minimal medium whereas ours was grown on a rich medium with a faster generation time. The average RNA content of cells grown on minimal medium is lower than that of cells grown on rich medium (Wehr & Parks, 1969). Thus cells grown on rich medium are required to produce ribosomal RNA at a higher rate, but from the same number of genes as in cells grown on minimal medium. Thus gene dosage could limit RNA synthesis in rich medium, whereas some other factor might limit in poor medium. Similar differences in the pattern of RNA synthesis through the cell cycle appear to exist in mammalian cells grown in rich and poor media (Pfeiffer & Tolmach, 1968; Scharff & Robbins, 1965; Klevecz & Stubblefield, 1967; Enger & Tobey, 1969; Stambrook & Sisken, 1972).

Our results suggest significant differences in the durations and times of occurrence of the increases in rRNA and poly(A)$^+$RNA synthetic rates. In three separate experiments, the increase in rRNA synthesis was earlier, and occupied a smaller portion of S phase, than the increase in poly(A)$^+$RNA synthesis (Fig. 9). The rRNA results suggest that the rRNA genes are replicated early in S phase, and quickly become available for transcription. The 140 rRNA genes of budding yeast are known to be clustered in groups of 10 to 32 (Cramer *et al.*, 1972) and 70% of the genes are on chromosome I (Finkelstein *et al.*, 1972; Øyen, 1973; Kaback *et al.*, 1973). This would suggest that chromosome I completes replication early during S phase. Schweitzer & Halvorson (1969) also reported that the ribosomal RNA genes replicate early during S phase, but a subsequent paper (Gimmler & Schweitzer, 1972) suggested that replication of the rRNA genes takes the whole of S phase.

At the time of the rise in the rate of rRNA synthesis, there was a reduction in the rate of processing of precursor rRNAs to the mature forms (Fig. 7). An explanation of this result is that the pool of ribosomal proteins is limited. When the rate of rRNA synthesis doubles, the rate of synthesis of ribosomal proteins does not double simultaneously, and processing of precursor rRNA to mature rRNA is retarded by lack of ribosomal proteins. A similar drop in the rate of processing occurs when protein synthesis is inhibited by cycloheximide (Udem & Warner, 1972). Later in the cycle, the rRNA precursor processing rate increased, perhaps because of an increased rate of synthesis of ribosomal proteins. The increase in the rate of poly(A)$^+$RNA synthesis (Fig. 8) coincided with the acceleration of the rate of precursor processing.

The comparatively large proportion of S phase over which the increase in rate of poly(A)$^+$RNA synthesis occurred is consistent with the idea that the poly(A)$^+$RNA must represent the transcription of a larger, more scattered proportion of the genome than the rRNA. As with rRNA, the poly(A)$^+$RNA results suggest that there is little delay between the replication of the DNA and the onset of transcription of both copies of each gene.

Knowing the number of ribosomal genes in *S. cerevisiae* (140 per haploid genome; Retel & Planta, 1968; Schweitzer *et al.*, 1969), we can attempt to calculate the number of genes coding for mRNA which are active in the cell. This calculation is based on the simplest interpretation of the changes in rate of poly(A)$^+$RNA synthesis through the cell cycle (Fig. 8), namely that "active" genes are transcribed, at a constant rate, throughout the cycle, with no switching, on or off, of genes. From Figure 2(a), we

derive approximate values for the percentage of total instantaneous RNA synthesis of 50 for polydisperse RNA (taken as total mRNA), 40 for rRNA precursors plus mature forms, and 10% is allowed for tRNA. The sizes of the primary gene products are taken to be $2{\cdot}5 \times 10^6$ daltons for rRNA (Udem & Warner, 1972) and an average of $0{\cdot}7 \times 10^6$ daltons for mRNA (Fig. 2(c)). We make the assumption that the polymerases transcribing the rRNA and mRNA genes move at the same rate and have the same spacing along the gene. The number of "active" mRNA genes per haploid genome is then

$$\frac{50}{40} \times \frac{2{\cdot}5}{0{\cdot}7} \times 140 = 625.$$

Intuitively, the figure of 625 active mRNA genes per haploid genome seems low for the number of proteins expected. This figure however, represents the *minimum* number of genes which, transcribed at maximum rate throughout the cell cycle would produce the labelling pattern observed. The cell cycle pattern of mRNA synthesis would not be significantly distorted if a small proportion of total mRNA synthesis was carried out by transcription of a large number of genes coding for mRNA, which were active for only part of the cycle or were transcribed at a slow rate.

(b) *Gene number, RNA synthesis and the control of balanced, exponential growth*

One reason for measuring rates of RNA synthesis through the cell cycle was to try to gain an understanding of the mechanisms controlling the balanced duplication of all cell components during each cell cycle. For ribosomal RNA, there is a straightforward link between gene number and the size of the ribosomal population. Replication of the ribosomal genes once per cycle leads to a doubling in the rate of rRNA synthesis once per cycle (Fig. 7). As ribosomal RNA can be assumed to be stable, this must lead to a doubling in rRNA content per cell in each cycle. We would expect rRNA to be accumulated linearly, with a doubling in the rate of accumulation at the time of the doubling in synthetic rate. Mitchison & Creanor (1969) have pointed out that such a linear rate-change pattern differs by a maximum of only 3% from an exponential increase in content which shows a doubling in the rate of accumulation over one cell cycle. The pattern of total RNA accumulation shown in Figure 6, which is close to exponential, could clearly also fit the linear rate-change model.

Our results for poly(A)$^+$RNA synthesis (Fig. 8) also suggest that most of these mRNAs are synthesised at a constant rate which doubles once per cycle during S phase. As the mRNA is unstable, this pattern of synthesis should result in a steady level of mRNA which should double after the doubling in rate of mRNA synthesis during S phase. Our data on changes in the content of polyadenylated RNA during the cell cycle (Fig. 8) are broadly in agreement with this prediction. From this pattern of mRNA content, we would expect that stable proteins should be accumulated at a constant rate which doubles once per cell cycle. The rate of total protein accumulation during the cell cycle in budding yeast has not been measured, but changes in the activities of individual enzymes have been studied in detail. It is surprising that not one of the more than thirty enzymes investigated is accumulated in a linear manner with a doubling in the rate of accumulation once per cycle. Most enzymes in budding yeast appear to increase periodically once per cycle, to give a characteristic step pattern of enzyme activity (reviewed by Halvorson *et al.*, 1971). This paradox might

be explained if the enzymes investigated were unstable: a doubling of the messenger content would lead to a doubled rate of enzyme synthesis and thus the establishment of a new steady-state enzyme concentration at twice the previous level. This explanation, however, can be ruled out for at least one enzyme, ornithine transaminase, which was found to be stable for at least one cell generation time (Sebastian *et al.*, 1973). Furthermore, the times of the steps in activity for different enzymes are not clustered at any particular stage in the cell cycle, but are distributed throughout the cycle (Tauro *et al.*, 1969). Clearly, periodic rises in enzyme activity do not bear any simple relationship to the doubling of the rate of poly(A)$^+$RNA synthesis shown here. The recent discovery in fission yeast of mechanisms controlling protein synthesis at the translational level (Creanor *et al.*, 1975; Fraser, 1975) raises the possibility that periodic enzyme synthesis in budding yeast might result from periodic translation rather than from periodic transcription.

This work was supported by the Medical Research Council and Science Research Council. We thank Mr R. Pitcairn and Miss A. Baker for technical assistance, and Prof. J. M. Mitchison for his interest in this work.

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J. Cell Sci. **21**, 497–521 (1976)
Printed in Great Britain

RATES OF SYNTHESIS OF POLYADENYLATED MESSENGER RNA AND RIBOSOMAL RNA DURING THE CELL CYCLE OF *SCHIZOSACCHAROMYCES POMBE*

WITH AN APPENDIX

CALCULATION OF THE PATTERN OF PROTEIN ACCUMULATION FROM OBSERVED CHANGES IN THE RATE OF MESSENGER RNA SYNTHESIS

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SUMMARY

The rates of polyadenylated messenger RNA and ribosomal RNA synthesis were measured in synchronously dividing cultures of fission yeast (*Schizosaccharomyces pombe*). Control asynchronous cultures, which had been exposed to the conditions used for preparing synchronous cultures, were investigated to check for effects of the synchronization procedure itself on RNA synthesis. After each period of DNA synthesis in synchronous culture, the rates of messenger and ribosomal RNA synthesis doubled, suggesting that gene number controls the rate of messenger and ribosomal RNA synthesis. This was confirmed by experiments with asynchronous, exponential-phase cultures in which DNA synthesis was inhibited by hydroxyurea. Both synchronous culture and hydroxyurea experiments suggested that there is a delay of 15 min (0·1 of the cell generation time) between replication of the DNA and transcription of both gene copies.

A pattern of protein accumulation was calculated from changes in the rate of polyadenylated messenger RNA synthesis during synchronous culture. This simulated pattern indicates that protein is accumulated linearly, with a doubling in the rate of accumulation once per cell cycle. The simulated pattern of protein accumulation is very similar to measurements previously reported by other workers of changes in activities of 3 enzymes in synchronous cultures.

It is suggested that the doubling of the rate of messenger RNA synthesis, as a consequence of the replication of the DNA once per cycle, provides the basis of a mechanism for control of the doubling of other cellular constituents during the cell cycle.

INTRODUCTION

Exponential growth of eukaryotic cells involves the doubling, during one cell cycle, of the DNA and all other cellular components, to produce 2 daughter cells identical to the parent. The mechanisms which control the balanced duplication of cellular components other than DNA are not understood. However, it is possible

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that the doubling of gene number during DNA replication may serve as a framework for the control of doubling of at least some other cellular components. It is therefore of interest to examine changes in the pattern of gene activity during the cell cycle by following rates of messenger RNA synthesis. Since the discovery that many eukaryotic messenger RNAs contain a polyadenylic acid sequence (Darnell, Wall & Tushinski, 1971; Lee, Mendecki & Brawerman, 1971; Edmonds, Vaughan & Nakazato, 1971; McLaughlin *et al.* 1973) and the development of methods for the isolation of this polyadenylated messenger RNA (Edmonds & Caramela, 1969; Aviv & Leder, 1972), it has become possible to study directly the rate of synthesis of a defined fraction of cellular messenger RNA.

We have measured changes in the rates of synthesis of messenger and ribosomal RNA in synchronously dividing cultures of the fission yeast *Schizosaccharomyces pombe*. This organism was chosen for study because the patterns of increase of several enzymes through the cell cycle have been studied in detail (Mitchison & Creanor, 1969). Knowledge of changes in the rate of messenger RNA synthesis during the cell cycle enables us to relate the changes in activity of these enzymes to gene number.

MATERIALS AND METHODS

Yeast and culture conditions

Schizosaccharomyces pombe Lindner, Strain N.C.Y.C. 132; A.T.C.C. 24751 was grown in a minimal medium EMM 2 (Mitchison, 1970) at 32 °C. Cultures of 200–2000 ml were grown in conical flasks on a rotary shaker operating at 220 rev. min^{-1}; 20-l. cultures were stirred by a motor-driven propellor. Cell growth was followed by measuring absorbance of cultures at 595 nm.

Uptake and incorporation of [2-^3H]adenine

One-millilitre samples of culture were added to 3·3 μCi [2-^{3}H]adenine (Radiochemical Centre, Amersham, U.K.; sp. act. 22 Ci. $mmol^{-1}$) and 1 μg non-radioactive adenine, and incubated for 5 min at 32 °C.

For adenine uptake measurements, 5 ml ice-cold water containing 100 μg ml^{-1} non-radioactive adenine were added to the incubation. The cells were collected by filtration on Whatman GF/A glass-fibre paper, washed 3 times with 5 ml cold adenine solution, then ^{3}H-radioactivity on the dried filters was measured by counting in 5 ml 0·5 % (w/v) butyl-PBD-toluene scintillator.

For measurement of adenine incorporation, 5 ml ice-cold 10 % (w/v) CCl_3COOH containing 100 μg ml^{-1} non-radioactive adenine were added to the incubation. The cells were collected by filtration, washed 3 times with 5 ml CCl_3COOH solution and radioactivity incorporated into acid-insoluble material determined by scintillation counting as above.

Synchronous cultures

Cells were harvested by filtration on Whatman No. 50 paper from 10–20 l. of exponential-phase culture grown to a cell concentration of 4×10^6 ml^{-1}. Small cells near the beginning of the cell cycle were selected by centrifugation on sucrose gradients (Mitchison & Vincent, 1965) and were re-inoculated into fresh EMM 2 to give a cell concentration of $1–1{\cdot}5 \times 10^6$ ml^{-1}. Progress of division in synchronous cultures was followed by monitoring the percentage of cells with a cell plate, which appears shortly before cell division.

Asynchronous control cultures were prepared to check for artifacts introduced by the

synchronization procedure. Cells were fractionated on sucrose gradients as above, then the gradients were mixed thoroughly before taking a sample containing cells at all stages of the cell cycle as inoculum for the asynchronous culture. Alternatively, cells from an asynchronous, exponential-phase culture were collected by filtration and resuspended in culture medium containing 27 % sucrose for 15 min before reinoculation.

Radioactive incubations

At 20-min intervals after inoculation of the synchronous or asynchronous control cultures, samples of 60–100 ml, containing $1–2 \times 10^8$ cells, were mixed with 3·3 μCi ml^{-1} [2-^{3}H]adenine for a 10-min pulse incubation. The adenine pool in fission yeast is expandable, and adenine uptake is extremely rapid (Cummins & Mitchison, 1967). In most experiments, the total adenine concentration of the pulse-incubation medium was raised to 1 μg ml^{-1} by addition of non-radioactive adenine. Under these conditions, the concentration of adenine in the medium did not change significantly during the pulse incubation: less than 10 % of the total adenine supplied was taken up by the densest cultures labelled.

As one approach to testing whether the rate of adenine uptake controlled the rate of incorporation, we also pulse-labelled one culture with 3·3 μCi ml^{-1} [2-^{3}H]adenine at a total adenine concentration of 0·03 μg ml^{-1}. Under these conditions, almost all of the adenine supplied was taken up by the cells during the pulse incubation, and uptake per ml culture remained constant during culture growth. All adenine incorporation results obtained under these labelling conditions were corrected to allow for this exhaustion of the adenine supply by dividing each incorporation value by the level of adenine uptake per cell for that pulse incubation. The effect of this procedure is to produce a curve for changes in adenine incorporation during culture growth which would result if adenine uptake per cell remained constant.

After the 10-min pulse incubation, the sample was chilled by addition of crushed ice. The cells were collected by filtration on Oxoid 6-cm diameter 0·45-μm pore-size filters, and resuspended in 5 ml ice-cold extraction medium (2 % (w/v) sodium tri-isopropylnaphthalene sulphonate in 10 mM NaCl; 50 mM Tris-HCl, pH 7·8) and stored frozen at −20 °C. Control experiments showed that incorporation of adenine into nucleic acids ceased immediately on chilling the culture.

The cell suspension was thawed at 0 °C. Ten-microlitre samples were dried on Whatman GF/A paper and counted in butyl-PBD-toluene scintillator as above to measure adenine uptake. Ten-microlitre samples were mixed with 5 ml ice-cold 10 % CCl_3COOH containing 100 μg ml^{-1} adenine. The cells were collected by filtration, washed 3 times with 5 ml CCl_3COOH solution and total adenine incorporation determined by scintillation counting as above. One-hundred-microlitre samples of cell suspension were diluted with 5 ml 0·9 % (w/v) NaCl. The cells were separated by sonication for 75 s in the M.S.E. sonicator, further diluted with saline, and cell number was determined using a Coulter Counter. Suspension of cells in extraction medium and freezing did not influence the cell number determination.

Extraction of nucleic acids

Total nucleic acid was extracted from the remainder of the cell suspension. The cells were broken by agitation with 2 g of glass beads (40 mesh) for 5 min in a Vibromixer (Shandon Scientific) and nucleic acids were extracted and deproteinized by successive phenol/chloroform treatments as explained in detail elsewhere (Fraser, Creanor & Mitchison, 1973; Fraser, 1975). The extracted nucleic acids were ethanol-precipitated and further purified by 3 cycles of reprecipitation from 0·5 % (w/v) sodium dodecyl sulphate in 150 mM sodium acetate pH 6·0 by 2·5 volumes of ethanol (Loening, 1969). The nucleic acids were stored as a precipitate in 80 % ethanol; 0·1 % sodium dodecyl sulphate; 30 mM sodium acetate, pH 6·0, at 4 °C.

A portion of the nucleic acid precipitate was collected by centrifugation at 2000 *g* for 5 min, then hydrolysed in 2·5 ml 0·5 N $HClO_4$ at 70 °C for 20 min. The ultraviolet absorption spectrum of the hydrolysate was measured in the Unicam SP800 recording spectrophotometer. Nucleic acid concentration was calculated from the absorbance at 260 nm, using a molar absorption coefficient of 12400 M^{-1} cm^{-1}, calculated for a solution of equal molar quantities of the 4 ribonucleotides at acid pH. In fission yeast, about 99 % of the total nucleic acid is RNA;

the absorption spectra therefore provided values for RNA per ml culture. The total yield of nucleic acid from each incubation was within the range 150 to 300 μg.

Measurement of DNA synthesis

Two equal aliquots of nucleic acid suspension were sedimented at 2000 *g* for 5 min. One was incubated with 0·5 ml 1 M KOH at 45 °C for 12 h to hydrolyse RNA. The other was washed with 1 ml 70% ethanol; 0·1 M NaCl, to remove sodium dodecyl sulphate, then dissolved in 0·1 ml 50 mM 2-(*N*-morpholino)ethanesulphonic acid, pH 7·0; 2 mM magnesium acetate. DNase (Sigma, electrophoretically purified) was added to 10 μg ml^{-1} and DNA was digested for 30 min at 20 °C. The sample was then digested with KOH as above.

Sixty per cent $HClO_4$ was added to each sample to reduce the pH to 1 and to precipitate $KClO_4$ as carrier, then 0·5 ml 10% CCl_3 COOH containing 100 μg ml^{-1} adenine was added. Acid-insoluble material was collected by filtration on Whatman GF/A glass-fibre paper, washed 3 times with ice-cold CCl_3COOH solution and radioactivity on the dried filters was determined as above. Radioactivity incorporated into DNA was calculated by subtracting the radioactivity of the DNase + KOH-digested sample from the radioactivity of the parallel, KOH-digested sample. At the mid-point of the period of DNA synthesis in synchronous cultures, about 90% of the KOH-resistant radioactivity was rendered acid-soluble by the DNase treatment.

The 'specific activity' of the DNA was expressed as DNA radioactivity per unit total nucleic acid in the sample. The specific activity could not be expressed as radioactivity per unit weight of DNA, as the amount of DNA in the samples was too small to measure accurately.

Synthesis of polyadenylated messenger RNA

Messenger RNA containing a polyadenylic acid sequence (poly(A)$^+$mRNA) was isolated from total nucleic acid using the specific binding of the poly(A) sequences, at high salt concentration, to the complementary homopolynucleotide oligodeoxythymidylic acid (oligo(dT)), immobilized on cellulose (Edmonds & Caramela, 1969; Aviv & Leder, 1972). Between 5 and 10 μg total nucleic acid were sedimented and dissolved in 0·5 ml binding buffer (400 mM NaCl; 10 mM Tris-HCl, pH 7·8; 1 mM EDTA; 0·2% sodium dodecyl sulphate) and mixed with 20 mg oligo (dT) cellulose (Collaborative Research Inc., Waltham, Mass., U.S.A.; binding capacity 100 A_{260} units poly(A) g^{-1}) for 30 min at 20 °C. The oligo (dT) cellulose was sedimented at 10000 *g* for 1 min, then washed 5 times with 0·5 ml of binding buffer to remove non-specifically bound nucleic acids. The oligo (dT) cellulose was then washed 4 times with 0·25 ml of elution buffer (Binding buffer minus NaCl) to release the poly(A)$^+$mRNA.

To determine the total radioactivity of the poly(A)$^+$mRNA, the combined elution buffer washes were dried at 70 °C in scintillation vials, then hydrolysed in 0·3 ml 0·5 M $HClO_4$ at 70 °C for 20 min. Seven millilitres of water-miscible scintillator (0·5% (w/v) butyl PBD; 40% (v/v) 2-methoxyethanol; 60% (v/v) toluene) were added and poly(A)$^+$mRNA radioactivity determined by scintillation counting. Correction of counts was by external standard counting, calibrated by addition of [^{3}H]toluene to a range of samples with different degrees of quenching. The 'specific activity' of the poly(A)$^+$mRNA was expressed as radioactivity per unit weight total nucleic acid fractionated on oligo(dT) cellulose, since the actual weight of poly(A)$^+$mRNA prepared by binding to oligo(dT) cellulose was too low to measure.

Examples of fractionations on sucrose gradients of fission yeast poly(A)$^+$mRNA samples are published elsewhere (Fraser, 1975). The poly(A)$^+$mRNA sediments on gradients to give a polydisperse distribution, ranging from 6 to over 30 S, with a broad peak around 12 S. There is no sign of contamination of the poly(A)$^+$mRNA by ribosomal or transfer RNAs.

Specific activity of ribosomal RNA

Ten microgrammes of total nucleic acid were fractionated on a 2·5% polyacrylamide gel for 2·5 h at 5 mA per gel, 8 V cm^{-1} gel length (Loening, 1967). The gels were scanned for absorbance at 265 nm in a Joyce Loebl Gel Scanner. The weight of ribosomal RNA present on the gels was found, in relative units, from the areas of the peaks for 25 S and 18 S rRNAs on

the absorbance scan. Peak area is linearly proportional to weight of RNA present (Fraser, 1971). The gel was frozen in solid CO_2 and sliced transversely at 1·0-mm intervals. Each slice was incubated with 0·3 ml 0·5 M $HClO_4$ at 70 °C for 20 min to hydrolyse the RNA; 7 ml of water-miscible scintillator were added and the radioactivity in each slice determined by scintillation counting. The total radioactivity of ribosomal RNA was calculated by addition of the radioactivities of the individual gel slices in the rRNA radioactivity peaks, after subtracting the background, polydisperse radioactivity. Radioactivity in the rRNA precursor was included in the total rRNA radioactivity. The specific activity of the rRNA was calculated as total rRNA radioactivity divided by the combined areas of the 25 and 18 S rRNA absorbance peaks. Ultraviolet absorption and radioactivity scans of gels which illustrate the measurement of rRNA amount and radioactivity by this method are published elsewhere (Fraser & Loening, 1974).

RESULTS

Artifacts caused by the synchronization procedure

It is essential when preparing synchronous cultures by a selection technique to check that the conditions of selection do not themselves induce changes which could be mistaken for cell-cycle related events. The disturbing factors to which yeast cells

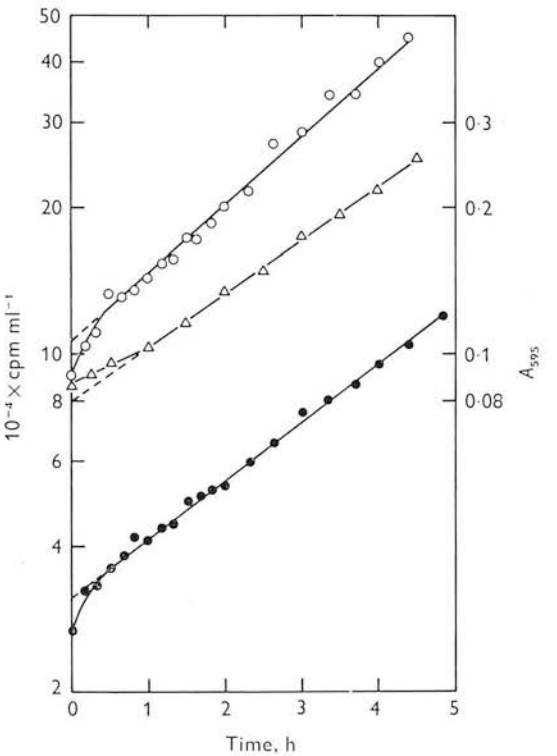

Fig. 1. Changes in optical density at 595 nm (△—△) and in rates of adenine uptake (○—○) and incorporation (●—●) ($10^{-4} \times$ cpm ml^{-1}) in an asynchronous culture of fission yeast. Cells from an exponential-phase culture were fractionated on a 10–40 % sucrose gradient (Mitchison & Vincent, 1965). The gradient was shaken after centrifugation and cells from all stages of the cell cycle re-inoculated into fresh medium. Samples were pulse-labelled for 5 min with 3·3 μCi ml^{-1} [2-^{3}H]adenine at a total adenine concentration of 1 μg ml^{-1}.

were exposed during selection synchronization included concentration by filtration, centrifugation, high sucrose concentration and re-inoculation into fresh culture medium. The two methods used to prepare asynchronous control cultures together exposed cells to all of these disturbing factors, but did not select cells by size before re-inoculation.

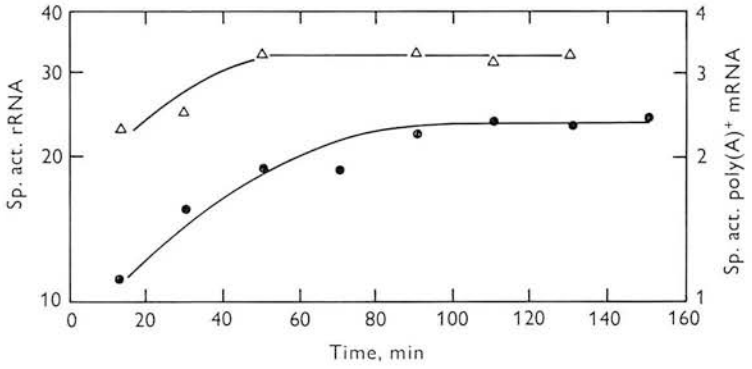

Fig. 2. Changes in specific activity of rRNA (△—△) (relative units) and poly(A)$^+$ mRNA (●—●) (10^{-4} × cpm μg^{-1} total nucleic acid) in an asynchronous culture of fission yeast. Cells from an exponential-phase culture were harvested and resuspended in medium containing 27% sucrose for 15 min, then re-inoculated into conditioned culture medium. Samples of the culture were pulse-labelled for 10 min with 3·3 μCi ml^{-1} [2-^{3}H]adenine; 1 μg ml^{-1} total adenine concentration, every 20 min during culture growth.

Fig. 1 shows changes in optical density, adenine uptake and incorporation rates in an asynchronous control culture inoculated with cells from a shaken sucrose density gradient. The rate of increase of optical density of the culture was low for the first 45 min, then optical density rose at a rate corresponding to a cell doubling time of 145 min, which is the time in normal exponential culture. The rates of adenine uptake and incorporation were low for the first 30 min, then rose exponentially until at least 4·5 h after inoculation. Thus the process of synchronization appeared to reduce growth of the culture for about the first 30 min after inoculation. There was no sign of any periodic changes in the rates of adenine incorporation or uptake during the period from 30 min to the end of the experiment. However, during this period the rates of increase of adenine uptake and incorporation were greater than the rate of increase of the optical density of the culture. In an asynchronous culture undergoing balanced exponential growth, the rates of increase should be identical. The explanation of this discrepancy is that adenine uptake rises with decreasing pH of the culture medium (R. S. S. Fraser, unpublished results). Fresh EMM 2, into which the cells were inoculated, has a pH of 5·3; during growth of the culture the pH falls to about 4·6.

The effects of the synchronization procedure on synthesis of specific types of RNA were also examined. Cells from an exponential-phase culture were concentrated, suspended at high concentration in 27% sucrose for 15 min, then re-inoculated into conditioned culture medium; 27% sucrose was used as it is the concentration of sucrose in the region of sucrose gradients from which cells are taken for inoculation

of synchronous cultures. The length of exposure to this sucrose concentration was the time required to load, centrifuge and fractionate a gradient. At intervals after re-inoculation of the asynchronous control culture, samples were pulse-labelled for 10 min with [2-^{3}H]adenine, and the specific activities of poly(A)$^+$mRNA and rRNA were determined. In asynchronous, exponentially growing cultures, the specific activities of pulse-labelled RNA fractions should remain constant with time. Fig. 2 shows that the specific activity of the rRNA rose for 30 min, then remained constant. This result is consistent with the changes in rate of adenine incorporation in the asynchronous control culture inoculated with cells from a shaken sucrose gradient (Fig. 1). The specific activity of the poly(A)$^+$mRNA did not become constant until 1·5 h after re-inoculation. The synchronization procedure therefore causes a depression of both rRNA and poly(A)$^+$mRNA syntheses; poly(A)$^+$mRNA synthesis takes much longer to recover. In some experiments we found that the initial depression of rRNA synthesis was much greater than of poly(A)$^+$mRNA, but recovery to a normal rate of rRNA synthesis still took only 30–60 min.

Changes in RNA synthesis during growth of synchronous cultures

Fig. 3 shows changes in cell number and cell plate index in a typical synchronous culture. The first division occurred between 2 and 3 h; the second between 4 and 5 h. Peaks of cell plate index at 2 h and 4 h 20 min were slightly in advance of the increases in cell numbers. The curve for DNA specific activity shows that DNA synthesis occurred in 2 periods, between 2 and 3 h, and between 4 and 5 h. This is consistent with previous results obtained by measuring total DNA by the diphenylamine method, which showed that the period of DNA synthesis (*S*-period) coincides with cell division (Bostock, Donachie, Masters & Mitchison, 1966; Mitchison & Creanor, 1971*a*). The 2 horizontal lines labelled *S*I and *S*II in Fig. 3C show the times of the first and second periods of DNA synthesis in the culture. These lines are reproduced in later figures showing changes in rates of RNA synthesis in the culture. Because of the imperfect synchrony of the culture, the duration of *S*-period in the entire culture will be longer than the duration of *S* in the individual cell.

Fig. 4A shows changes in total nucleic acid per ml culture during growth of the synchronous culture. There is considerable scatter of points, as the yield of nucleic acids by the phenol-detergent extraction method is variable. This is entirely a consequence of variable degrees of breakage of cells during the extraction. Total nucleic acid per ml culture increased approximately exponentially, with a doubling time equal to the cell generation time.

Fig. 4B shows that rates of uptake and incorporation of [2-^{3}H]adenine changed in a periodic manner during growth of the synchronous culture. The portion of the curves drawn in broken lines at the start of culture indicates those points which were probably depressed by the effects of the synchronization conditions (Fig. 1). While the pattern of periodic change is the same for uptake and incorporation, we consistently found that the timings of the changes were different, with incorporation changing about 15 min before uptake.

Changes in the specific activities of pulse-labelled rRNA and poly(A)$^+$mRNA in

2 synchronous cultures are shown in Fig. 5. The results are expressed as specific activities to eliminate variation arising from differences in efficiency of extraction of nucleic acids. Poly(A)$^+$mRNA specific activity was fairly constant until the middle of the first *S*-period, rose during the second half of the first *S*-period, then declined

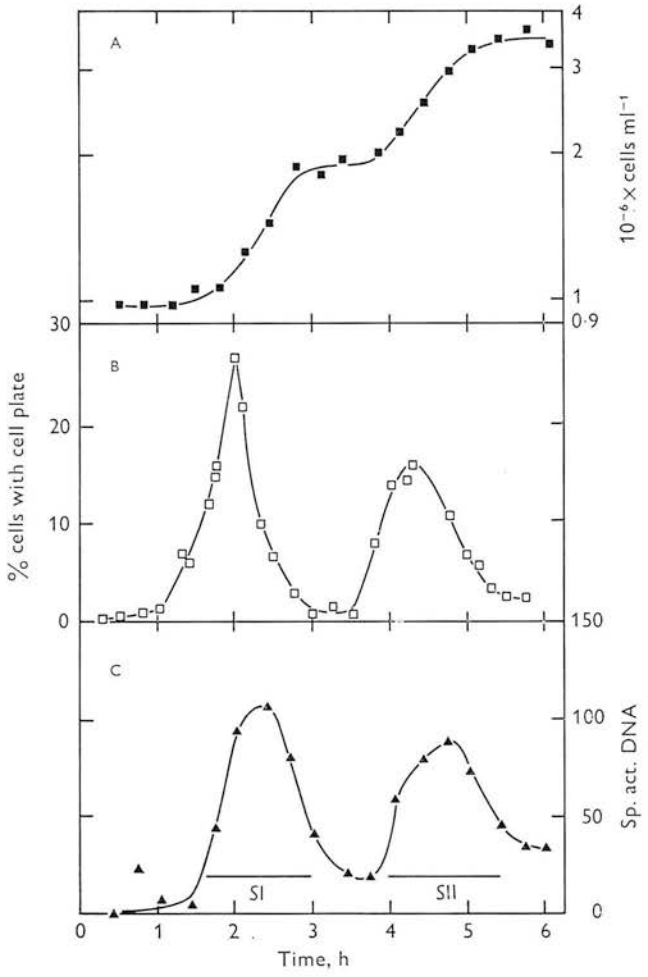

Fig. 3. Changes in A, cell number per ml; B, percentage of cells with a cell plate, and C, specific activity of DNA (cpm μg^{-1} total nucleic acid), in a synchronous culture. Samples of culture were labelled for 10 min with 3·3 μCi ml^{-1} [2-^{3}H]adenine; 1 μg ml^{-1} total adenine concentration. The horizontal bars *S*I and *S*II in C indicate the durations of the first and second periods of DNA synthesis in the culture.

between the first and second *S*-periods. A second rise in poly(A)$^+$mRNA specific activity occurred late in the second S-period. rRNA specific activity was initially very low in both cultures, but rose rapidly, then declined during most of the first *S*-period. Late in the first *S*-period, rRNA specific activity rose, then declined again between the first and second *S*-periods, and during most of the second *S*-period. Late in the second *S*-period the specific activity of rRNA again rose. Each rise in rRNA

specific activity during *S* was later than the corresponding rise in the specific activity of poly(A)$^+$mRNA.

The culture shown in Fig. 5A was labelled at a total adenine concentration of 1 μg ml^{-1}; that shown in Fig. 5B with 0·03 μg ml^{-1}, and the results were corrected for exhaustion of adenine during the pulse as explained in Materials and methods. It is clear that the patterns of changes in specific activities of RNA in the 2 cultures were similar, despite the different labelling conditions.

The rises in specific activities of pulse-labelled poly(A)$^+$mRNA and rRNA after commencement of DNA synthesis suggest a connexion between DNA content and rate of RNA synthesis. However, the expression of the data as specific activities,

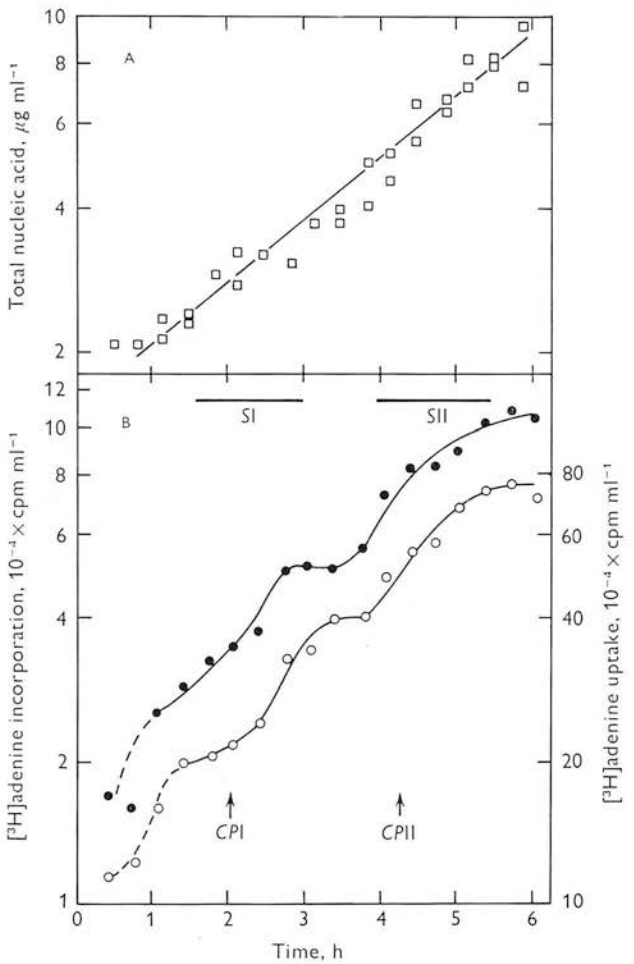

Fig. 4. Changes in A, total phenol-detergent extracted nucleic acid per ml culture; and B, incorporation (●—●) and uptake (○—○) (10^{-4} × cpm ml^{-1}) of [2-^{3}H]adenine during growth of a synchronous culture. Samples of culture were pulse-labelled for 10 min with 3·3 μCi ml^{-1} [2-^{3}H]adenine and 1 μg ml^{-1} total adenine concentration. *S*I and *S*II indicate the times of the first and second periods of DNA synthesis; CP I and CP II show times of first and second peaks of cell plate index (from Fig. 3).

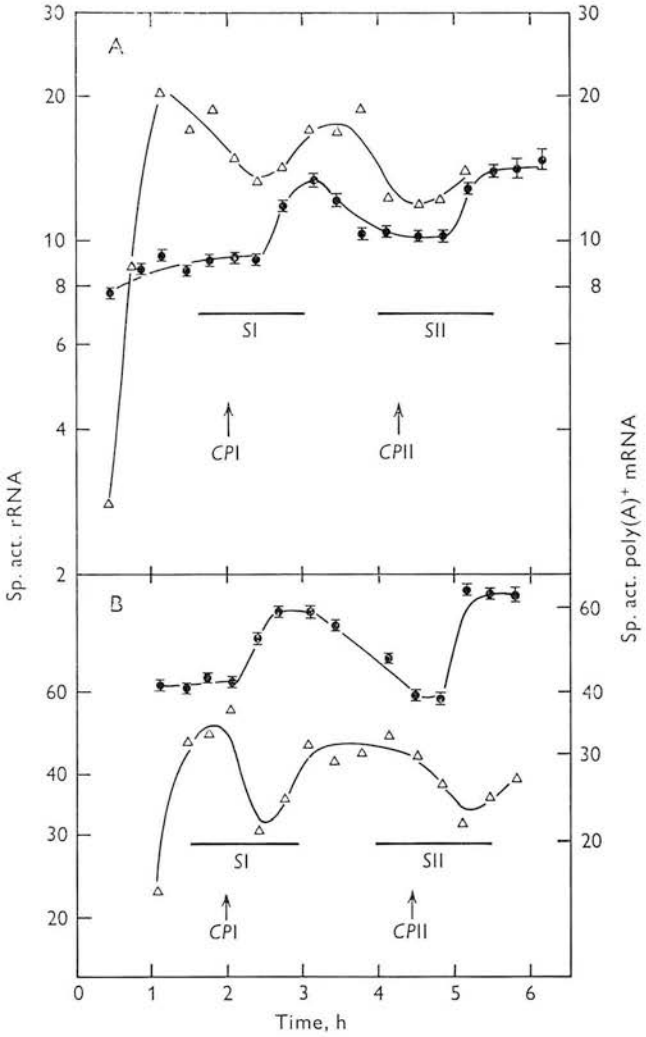

Fig. 5. Changes in specific activities of rRNA (△—△) (relative units) and poly(A)$^+$ mRNA (●—●) ($10^{-2} \times$ cpm μg^{-1} total nucleic acid) during synchronous cultures of fission yeast. The vertical lines and cross-bars on the poly(A)$^+$mRNA points show the standard errors of the means. Each point is the mean of 6 determinations by oligo(dT)-cellulose fractionation. *S*I and *S*II show the times of the first and second periods of DNA synthesis in each culture, determined as shown in Fig. 3c. CPI and CPII indicate the times of the first and second peaks of cell plate index. The results shown in A are from a culture pulse-labelled with 3·3 μCi ml^{-1} [2-^{3}H]adenine and a total adenine concentration of 1 μg ml^{-1}. In B, samples of culture were pulse-labelled with 3·3 μCi ml^{-1} [2-^{3}H]adenine and a total adenine concentration of 0·03 μg ml^{-1}. The results shown in B have been corrected for exhaustion of adenine in the pulse medium by dividing each incorporation value by the level of adenine uptake per cell at the time of the pulse.

while avoiding variation arising from different efficiencies of extraction of nucleic acids, does not make immediately apparent the full relationship of the rate of RNA synthesis to the occurrence and timing of DNA synthesis. To investigate this relationship, the primary data can be transformed in 2 ways. Firstly, the rate of poly(A)$^+$ mRNA synthesis per ml culture can be calculated by multiplying the specific activity (cpm μg^{-1} total nucleic acid) by the value for total nucleic acid per ml culture at the

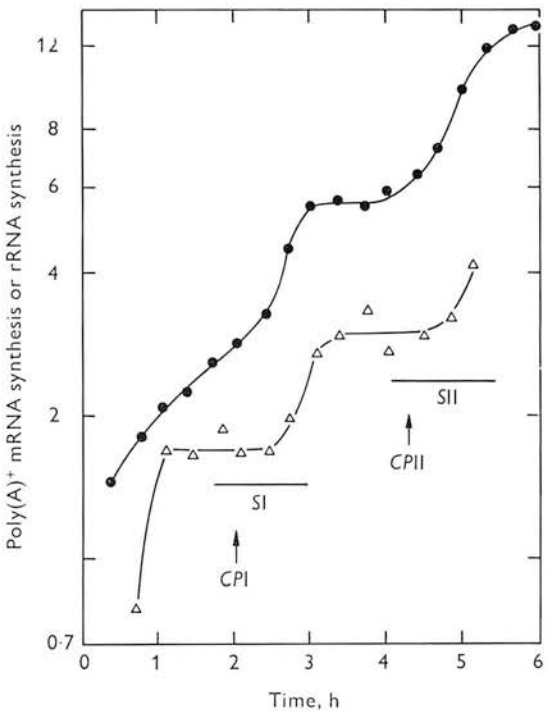

Fig. 6. Changes in the rate of rRNA synthesis (△—△) (relative units) and poly(A)$^+$-mRNA synthesis (●—●) (10^{-3} × cpm) per ml culture during synchronous culture, calculated from changes in specific activities of rRNA and poly(A)$^+$mRNA shown in Fig. 5A and changes in total nucleic acid extracted per ml culture shown in Fig. 4A. *S*I and *S*II show the times of the first and second periods of DNA synthesis; CP I and CP II, the times of the first and second peaks of cell plate index (from Fig. 3C, B, respectively).

time of the pulse incubation. Specific activity data for rRNA can be similarly treated to give rate of rRNA synthesis per ml culture. We have taken values for total nucleic acid per ml culture from the line calculated to give the best fit to the experimental points in Fig. 4A, rather than using the actual determined values. This avoids variation from differences in the efficiency of extraction of nucleic acids.

Fig. 6 shows changes in the rates of poly(A)$^+$mRNA and rRNA synthesis per ml culture, calculated from the changes in specific activities in the synchronous culture shown in Fig. 5A. The initial rate of rRNA synthesis was very low, a consequence of the transitory depression of rRNA synthesis by the conditions of synchronization (Fig. 2). Poly(A)$^+$mRNA synthesis was also depressed in the early stages of culture

by the synchronization procedure: the extent of this depression will be shown more clearly below.

Following the commencement of the first *S*-period, the rates of synthesis of both rRNA and poly(A)$^+$mRNA doubled. Between the first and second *S*-periods, the rates of synthesis of both types of RNA remained constant. After the beginning of the second *S*-period, the rate of poly(A)$^+$mRNA synthesis per ml culture again doubled; rRNA synthesis also increased. The doubling of rates of RNA synthesis during *S*-periods suggests that gene number controls the rate of RNA synthesis.

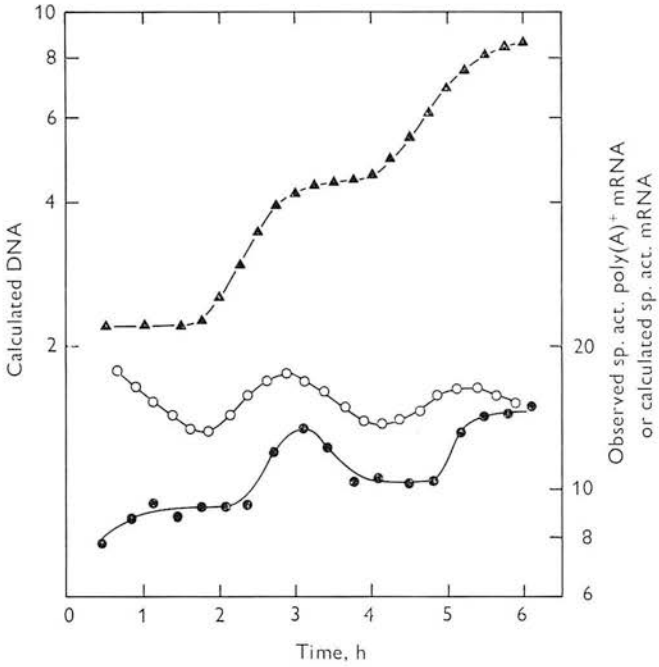

Fig. 7. Changes in DNA content (relative units) per ml culture (▲—▲), calculated from the DNA specific activity changes during synchronous culture shown in Fig. 3C. (●—●), observed changes in poly(A)$^+$mRNA specific activity ($10^{-3} \times$ cpm μg^{-1} total RNA) during synchronous culture (from Fig. 5A); (○—○), calculated changes in mRNA specific activity (relative units) where the rate of mRNA synthesis is controlled only by gene number.

A more sensitive test of the relationship of poly(A)$^+$mRNA synthesis to the occurrence and timing of DNA synthesis is obtained by calculating a curve for changes in mRNA specific activity which would be produced if gene number alone were controlling the rate of RNA synthesis. From the changes in DNA specific activity during synchronous culture (Fig. 3C), we obtain a curve for DNA synthesis per ml culture by multiplying the specific activity (cpm μg^{-1} total nucleic acid) by total nucleic acid ml^{-1} at the time of the pulse incubation (from Fig. 4A). Integration of the curve for DNA synthesis per ml culture over successive time increments gives a curve for DNA content per ml culture. The amount of DNA at time 0 is taken to equal the increment in DNA during the first *S*-period. This curve is shown in Fig. 7. If the rate of mRNA

synthesis is controlled only by gene number, the amount of mRNA synthesized in any time period is proportional to the average DNA content during that period. Dividing this figure for synthesized mRNA by the total nucleic acid per ml culture at this time gives a curve of changes in mRNA specific activity where mRNA synthesis is limited only by gene number. This theoretical specific activity curve (Fig. 7) rises during periods of DNA synthesis, and falls between *S*-periods, as total nucleic acid accumulates. The amplitude of the periodic fluctuation gradually decreases, reflecting the gradual loss of synchrony of division and DNA synthesis in the culture.

It is clear that the calculated changes in mRNA specific activity are broadly similar to the experimentally measured changes in poly(A)$^+$mRNA specific activity (Fig. 7). However, there are 2 important differences between the 2 curves. In the first 1·5 h of culture, the pattern of changes in the experimental data is very different from that of the calculated data; the experimental values are lower than expected. This is a consequence of the depression of early poly(A)$^+$mRNA synthesis by the synchronization procedure (Fig. 2). Secondly, while thereafter the periodic fluctuations of experimental and calculated specific activities were similar, attempts to superimpose the calculated data on the experimental data consistently showed that the changes in the experimental curve were about 15 min later than the corresponding changes in the calculated curve. This suggests that while the rate of poly(A)$^+$mRNA synthesis may be basically regulated by gene number, there is a short delay between the replication of the DNA and the transcription of both copies of the gene. An alternative explanation is that DNA which is actively transcribed may be replicated late in the *S*-period, but this is made unlikely by the evidence to be discussed below.

RNA synthesis after inhibition of DNA synthesis

The results described above suggest a dependence of RNA synthesis rate on gene number, but do not establish a causal relationship. Further evidence for the control of the rate of poly(A)$^+$mRNA synthesis by gene number, and for the 15-min delay between DNA replication and transcription of both gene copies, comes from experiments with asynchronous, exponential-phase cultures in which DNA synthesis was inhibited. The rate of poly(A)$^+$mRNA synthesis in control cultures rose exponentially, at the same rate as cell growth (Fig. 8). Twelve millimolar hydroxyurea was added to one half of the culture to inhibit DNA synthesis (Mitchison & Creanor, 1971*b*). Within 5 min of adding the inhibitor, the specific activity of DNA after a pulse-label with [2-^{3}H]adenine was one-quarter that in the control, and no significant pulse-labelling of DNA was detectable by 10 min after addition of hydroxyurea. The rate of poly(A)$^+$mRNA synthesis in hydroxyurea-treated cells rose at the same rate as in control cultures for 15–20 min, then became stable for at least 1 h. By 100 min after addition of hydroxyurea, there was again a small rise in the rate of poly(A)$^+$ mRNA synthesis. The rate of uptake of adenine by hydroxyurea-treated cells rose at the same rate as in control cells for about 50 min, then became constant. This suggests that the effects of hydroxyurea on labelling of poly(A)$^+$mRNA were not merely a consequence of an effect of the inhibitor on isotope uptake, but were an effect on poly(A)$^+$mRNA synthesis. Overall cell growth, measured by increase in the optical

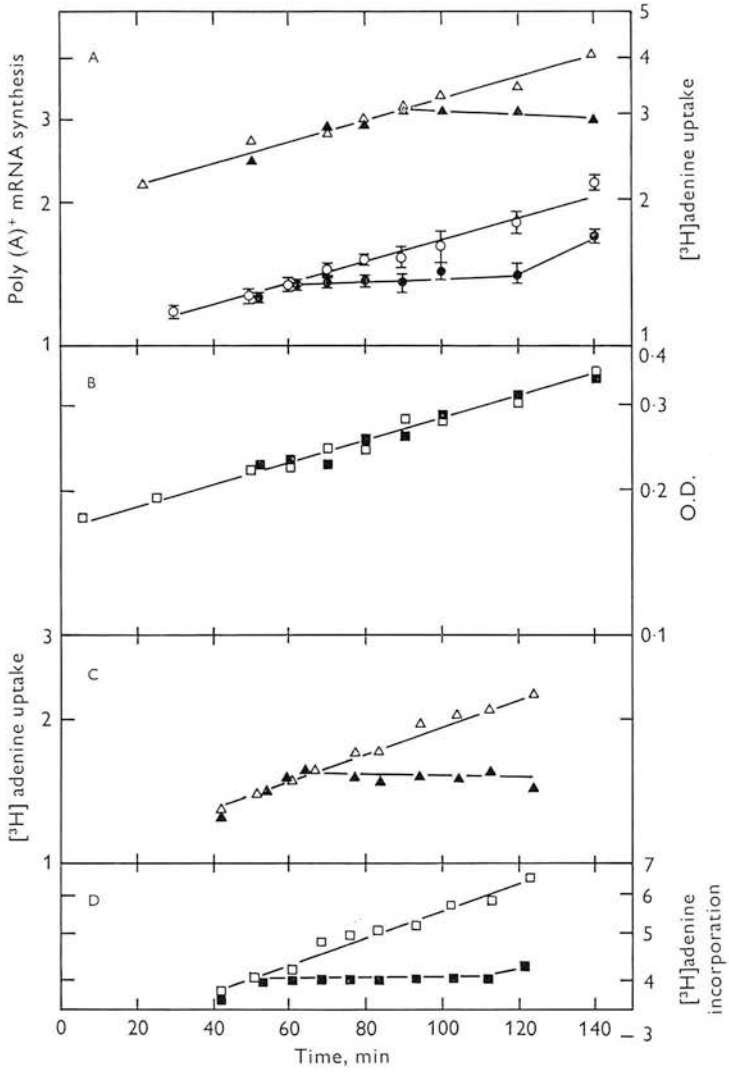

Fig. 8. A, changes in the rates of adenine uptake (△, ▲) ($10^{-5} \times$ cpm ml^{-1}) and poly-(A)⁺mRNA synthesis (○, ●) ($10^{-4} \times$ cpm ml^{-1}) in asynchronous, exponential cultures of fission yeast; 12 mM hydroxyurea was added to one half of the culture at 40 min. Results from the control culture are shown (△, ○); and from the hydroxyurea-treated culture (▲, ●). Horizontal bars and vertical lines on the points for poly(A)⁺mRNA synthesis indicate the standard errors of the means. Each point is the mean of 6 determinations by oligo(dT) cellulose fractionation. B, changes in optical density at 595 nm, of the control culture (□) and the hydroxyurea-treated culture (■). C, changes in total adenine uptake ($10^{-5} \times$ cpm ml^{-1}), and D, adenine incorporation ($10^{-4} \times$ cpm ml^{-1}), in a separate experiment from A and B: (△, □), control cultures; (▲, ■), cultures to which 12 mM hydroxyurea was added at 40 min.

density of the culture, was not affected by hydroxyurea within the time of the experiment.

The change from an exponentially increasing rate of poly(A)$^+$mRNA synthesis to a constant rate after addition of hydroxyurea to inhibit DNA synthesis confirms that synthesis of poly(A)$^+$mRNA is controlled by gene number. The 15–20-min delay between addition of the inhibitor and the onset of a constant rate of poly(A)$^+$mRNA synthesis again suggests that there is a short delay between gene replication and a doubled rate of transcription. The late recovery in rate of poly(A)$^+$mRNA synthesis in hydroxyurea-treated cells probably occurred because the inhibition of DNA synthesis by hydroxyurea in fission yeast is not permanent (Mitchison & Creanor, 1971*b*).

In a separate experiment, the rate of total RNA synthesis became constant 10–15 min after addition of hydroxyurea (Fig. 8D). In this case the adenine uptake rate increased at the same rate as in control cells for 30 min after addition of hydroxyurea, then became stable (Fig. 8C).

DISCUSSION

Cell cycle analysis

The measurement of changes in the rate of RNA synthesis during the cell cycle using selection-synchronized cultures has one disadvantage. The synchronization procedure itself seriously affects RNA synthesis, especially poly(A)$^+$mRNA synthesis, and these artifacts must be investigated before interpreting changes in relation to the cell cycle. We have shown that the first cell cycle in synchronous culture, that is the period from inoculation to the first division, is subject to these artifacts. Thereafter, the changes observed can be taken to be cell cycle-related events.

A better approach to investigation of changes in RNA synthesis during the cell cycle would be to pulse-label an asynchronous, exponentially growing culture, then separate cells by cell volume, hence stage in the cell cycle, by zonal centrifugation (Carter, Sebastian & Halvorson, 1971). We have used this method in a parallel study of RNA synthesis during the cell cycle in the budding yeast *Saccharomyces cerevisiae* (Fraser & Carter, 1976). However, for our study of fission yeast, synchronous cultures were chosen, because previous investigations of changes in enzyme activity were carried out with synchronous cultures; it was our aim to correlate gene number, messenger RNA synthesis and patterns of enzyme activity.

Throughout these experiments, we have assumed that the rate of incorporation of [2-^{3}H]adenine into RNA is a measure of the rate of RNA synthesis, and that changes in the rate of incorporation do not merely reflect changes in the rate of adenine uptake or precursor pool specific activity. We have several reasons for concluding that incorporation rate is a true measure of synthesis. Firstly, where incorporation and uptake rates were followed in synchronous cultures or after addition of hydroxyurea, both uptake and incorporation displayed the same overall pattern of changes. However, changes in uptake rate were always later than corresponding changes in rate of incorporation. This suggests that uptake rate is determined by the rate of incorporation,

and not *vice versa*. Secondly, in synchronous cultures, the rise in specific activity of poly(A)$^+$mRNA during *S*-period was consistently earlier than the rise in rRNA specific activity (Fig. 5). It is difficult to reconcile this difference with a situation where incorporation is controlled by uptake rate or precursor pool specific activity, unless there are separate precursor pools for mRNA and rRNA. Thirdly, the size of the adenine-labelled acid-soluble pool, as a proportion of cell dry mass, remains essentially constant during the cell cycle (Mitchison, Cummins, Gross & Creanor, 1969). Finally, the same basic patterns of changes in specific activities of poly(A)$^+$mRNA and rRNA were found when synchronous cultures were labelled with high or low adenine concentrations. At the high adenine concentration, the amount of uptake depended on the activity of the adenine uptake mechanism of the cells. Results from the culture labelled at the low adenine concentration were expressed so as to show the incorporation pattern in an artificial situation where uptake per cell remained constant during culture. The effect of this is to nullify any effects of changes in uptake rate on the rate of incorporation.

rRNA synthesis during the cell cycle

In synchronous cultures, the specific activity of pulse-labelled rRNA rose after commencement of the period of DNA synthesis. The rate of rRNA synthesis per ml culture doubled at the end of the first *S*-period, and began to rise again late in the second *S*-period. Experiments with hydroxyurea, showing that the rate of total RNA synthesis became stable after inhibition of DNA synthesis, suggested that the doubling during synchronous culture of the rate of rRNA synthesis was a result of the increase in DNA content. These results confirm earlier experiments with *S. pombe* by Wain & Staatz (1973) which showed a rise in the rate of labelling of total, long-pulse-labelled RNA at about the time of DNA synthesis.

The rRNA in *S. pombe* in exponential growth is essentially stable (Fraser, 1975). Therefore a doubling of the rate of rRNA synthesis once per cycle will lead to a doubling of rRNA content over one cell cycle. This provides a basis for control of the doubling of the size of the ribosomal population in each cell cycle.

The rise in specific activity of rRNA during *S* consistently occurred later than the rise in specific activity of poly(A)$^+$mRNA. This may mean that the DNA coding for rRNA is replicated very late during *S*. The alternative explanation, that there is a long lag between replication of rDNA and transcription of both copies, is made unlikely by the rapid onset of a steady rate of total RNA synthesis after addition of hydroxyurea. In contrast to these results, we found in *Saccharomyces cerevisiae* that the rate of rRNA synthesis doubled very early in *S*, suggesting that in budding yeast the rDNA is replicated early in *S* and is immediately available for transcription (Fraser & Carter, 1976).

Poly(A)$^+$mRNA synthesis during the cell cycle

Our results show that shortly after the commencement of the period of DNA synthesis, the rate of poly(A)$^+$mRNA synthesis per ml culture doubles. A causal relationship between DNA content and the rate of poly(A)$^+$mRNA synthesis was

confirmed by the experiment in which DNA synthesis was inhibited by hydroxyurea. In *Saccharomyces cerevisiae*, we have also found that the rate of poly(A)$^+$mRNA synthesis doubles during the *S*-period (Fraser & Carter, 1976).

We conclude that the rate of poly(A)$^+$mRNA synthesis is controlled by gene number. This offers the basis of a mechanism for control of the doubling of cellular components once per cell cycle. A doubling of the rate of messenger synthesis once per cycle should lead, in the simplest case, to a doubling in the rate of enzyme accumulation once per cycle, and hence to a doubling in the metabolic capacity of the cell. The simplest interpretation of our data for poly(A)$^+$mRNA synthesis per ml culture is that an 'active fraction' of the genome is transcribed at a constant rate throughout the cell cycle; doubling of the numbers of these genes during *S* doubles the rate of messenger RNA synthesis. This simple pattern would account for the bulk of total messenger synthesized in the cell, but could obscure a more complex situation where a small proportion of total mRNA synthesis was from other genes which were transcribed periodically or at varying rates through the cycle. We are also unable to estimate what proportion of the total genome is represented by the 'constantly transcribed fraction' of our simple model.

Some further qualifications must be made to the model relating gene number and rate of mRNA synthesis to doubling of cell components. The model is based on measurements of the rate of synthesis of polyadenylated mRNA, which probably does not represent the entire mRNA metabolism of yeast (McLaughlin *et al.* 1973). The poly(A)$^+$mRNA we studied was prepared from whole cell nucleic acid, and thus contained not only cytoplasmic mRNA, but also nuclear precursors and any special stored or transit forms. Any cell cycle-specific variation in processing or storage could complicate the situation. Furthermore, we have examined cells growing at close to their maximum growth rate. It is possible that in cells growing at a much slower growth rate, factors other than gene dosage might be limiting, in which case a different mechanism for the co-ordination of balanced duplication of cellular components during the cell cycle might exist. Differences in the pattern of rRNA synthesis during the cell cycle have been reported for mammalian tissue culture cells growing at different growth rates (Pfeiffer & Tolmach, 1968; Scharff & Robbins, 1965; Klevecz & Stubblefield, 1967; Enger & Tobey, 1969).

Studies on the dependence of poly(A)$^+$mRNA synthesis on gene number using synchronous cultures or hydroxyurea both indicated that there is a lag of 15–20 min between DNA replication and an increase in the rate of transcription. This lag might be caused by physical unavailability of the new DNA for transcription, or by temporary limitation, by some factor other than gene number, of the rate of transcription. It is interesting that when cells are grown at a slightly faster growth rate, in malt extract broth, there is no lag between DNA replication and a doubled rate of poly(A)$^+$mRNA synthesis (R. S. S. Fraser, unpublished results).

Gene number, mRNA synthesis and patterns of enzyme activity in synchronous cultures

The best test of whether the pattern of changes in rate of poly(A)$^+$mRNA synthesis we have observed during the cell cycle is meaningful in the control of cell growth is

to examine whether it can account for observed changes in enzyme activities during the cell cycle. We have calculated the pattern of protein accumulation which would be produced from the changes in rate of poly(A)$^+$mRNA synthesis during synchronous culture shown in Fig. 6. The Appendix describes how the protein accumulation data were calculated, and how the goodness of fit of different curves to the calculated points was tested. Fig. 9 shows the calculated protein-accumulation data. The curve which gave the best fit to the points was for linear accumulation of protein, with a doubling in the rate of accumulation at 2 h 50 min and 5 h 15 min after inoculation.

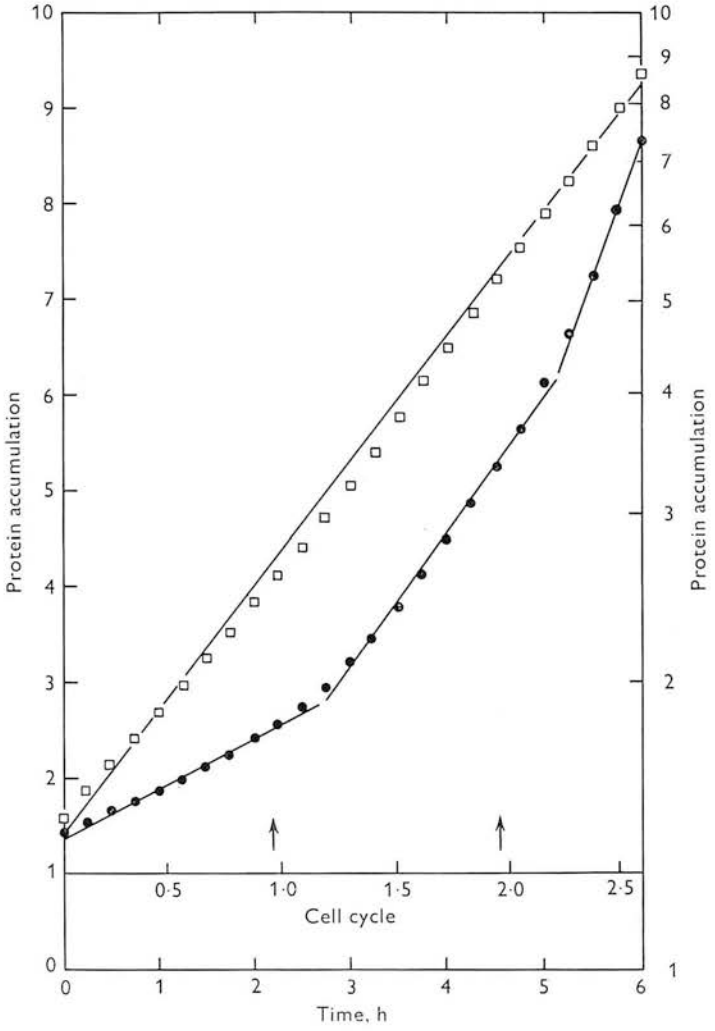

Fig. 9. The pattern of protein accumulation (relative units) during growth of a synchronous culture, calculated from changes in the rate of poly(A)$^+$mRNA synthesis during culture as explained in the appendix. The data are plotted on linear (●—●) and logarithmic (□—□) scales. The cell cycle scale (1·0 unit equals one cell generation time) is drawn from the cell number data of Fig. 3A; 1·0 and 2·0 represent the mid-points of the first and second divisions respectively. The 2 arrows indicate the times of the mid-points of the first and second periods of DNA synthesis (from Fig. 3C).

Taking the cell cycle from one division to the following division as 1·0 unit, these 2 rate-change points occur at 0·2 of the second and third cell cycles after inoculation. We have not considered a possible rate change at 0·2 in the first cycle after inoculation, as there would be too little data available before the rate change to establish the initial rate.

The pattern of protein accumulation calculated from the rates of poly(A)$^+$mRNA synthesis is very similar to the patterns of increase in enzyme activities measured in similar synchronous cultures by Mitchison & Creanor (1969). They found that sucrase, acid phosphatase and alkaline phosphatase all increased in activity linearly; the rate of synthesis of each enzyme doubled at close to 0·2 in each cell cycle.

The similarity of the calculated protein accumulation pattern (Fig. 9) to observed changes in enzyme activities (Mitchison & Creanor, 1969) suggests that the measured changes in the rate of poly(A)$^+$mRNA synthesis are meaningful in the control of cell growth. These results lend support to our suggestion that the balanced duplication of cell components once per cycle may be largely controlled by constant transcription of a rate-limiting number of genes coding for mRNA. The explanation of observed patterns of enzyme increase purely by consideration of changes in mRNA synthesis further suggests that the content or availability of ribosomes does not play a part in the regulation of protein synthesis in this case.

Our data and calculations show that the doubling in rate of protein accumulation at 0·2 of the cell cycle is a consequence of the doubling of gene number during *S.* The data explain the paradox noted by Mitchison & Creanor (1969): that the DNA synthesis in *S. pombe* occurs at about 0·9 to 1·0 of the cell cycle, but the rate-doubling point for their enzymes was at 0·2 in the following cycle. This delay is in contrast to bacterial systems, where replication of the structural gene appears to be followed immediately by a doubled rate of enzyme accumulation (Kuempel, Masters & Pardee, 1965; Donachie, 1965; Helmstetter, 1968; Donachie & Masters, 1969). The delay of 0·2–0·3 of a cell cycle in *S. pombe* can be ascribed to 2 factors: a delay of 15–20 min (about 0·1 of a cycle) which remains to be explained, between replication of the DNA and a doubled rate of transcription, and a delay of up to 30 min (0·2 of a cycle) between the doubling of the rate of mRNA synthesis and the establishment in the cell of a sufficiently increased messenger content to raise the rate of protein synthesis by a detectable amount. It is clear from the calculated curves for messenger RNA accumulation (Appendix; Figs. 10, 11, pp. 519–520) that the approach to a doubled messenger content after doubling of the rate of transcription is slow.

We have compared a simulation of protein accumulation, derived from measurements of synthesis of a major fraction of mRNA, with experimental data for individual enzymes. It will be interesting to compare the simulated protein accumulation pattern with experimental results on total protein accumulation or synthesis during synchronous culture when such data become available. Total protein accumulation will represent the aggregate effects of all protein-synthesis control mechanisms, operating at transcriptional and translational levels (Creanor, May & Mitchison, 1975; Fraser, 1975) as well as turnover effects. It is possible that patterns of total protein accumulation may diverge from the pattern simulated from data of poly(A)$^+$mRNA synthesis.

This work was supported by a grant from the Medical Research Council. F. Moreno thanks the European Molecular Biology Organization for a short-term fellowship. We thank Dr L. Errington for technical assistance, and Professor J. M. Mitchison for his interest in this work.

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(*Received* 30 *December* 1975)

APPENDIX: CALCULATION OF THE PATTERN OF PROTEIN ACCUMULATION FROM OBSERVED CHANGES IN THE RATE OF MESSENGER RNA SYNTHESIS

We will calculate the pattern of protein accumulation which would arise from the changes in rate of messenger RNA synthesis measured during growth of a synchronous culture (Fig. 6). The calculation has 2 steps: calculation of changes in messenger content per ml culture during growth, and calculation of a curve for protein accumulation from these changes in messenger content.

Calculation of changes in messenger content

To calculate changes in messenger RNA content from changes in the rate of mRNA synthesis, we require to know the average half-life time ($t_{0.5}$) of yeast poly(A)$^+$mRNA, and we have to be able to express the measured rate of mRNA synthesis in the same units as the rate of degradation. The mean $t_{0.5}$ of fission yeast poly(A)$^+$mRNA has been measured by 2 independent methods, which both give a value of 40–45 min (Fraser, 1975). Assuming that at the start of a synchronous culture, the messenger

RNA content is 100 units ml^{-1}, we will calculate changes in content resulting from degradation and from changes in the relative rate of synthesis during culture.

Considering first only degradation, the amount of the original 100 units which degrades in a given time interval t is obtained from Equation (1):

$$n_t = n_o e^{-rt}, \tag{1}$$

where n_o is the starting population, n_t is the population remaining after time t, and r is the rate constant. The equation holds for decay which follows first-order kinetics; the decay of fission yeast poly(A)$^+$mRNA does substantially show first order kinetics (Fraser, 1975). For $t = t_{0.5} = 40$ min, $n_o = 2n_t$ and $r = -0.0173$ min^{-1}. We will consider mRNA content at 15-min intervals during culture; the fraction of n_o degraded after 15 min is 0·229.

In a situation where synthesis also occurs, to find the net change in mRNA content over a 15-min time interval we must in addition consider:

(*a*) *The mRNA synthesized during the* 15 *min.* Let the unit of mRNA synthesis be s, and let $s = -r$. Let the relative rate of mRNA synthesis (shown in Fig. 6) at time 0 in the synchronous culture be m, and at the mid-point of the 15-min interval of synchronous culture growth being considered be m'. k is a constant, the significance of which will be explained later. Then the average rate of mRNA synthesis during the 15-min interval is $ks.m'/m$ min^{-1}, and this rate is in the same units as the rate of degradation r. Thus the amount of mRNA synthesized in the 15-min time interval is $15\ ks.m'/m$.

(*b*) *The degradation of messenger which was synthesized during the* 15*-min time interval must be subtracted.* Taking the average content of newly synthesized mRNA during the interval as $\frac{15}{2}.ks.m'/m$, the amount of the newly synthesized mRNA degraded is calculated as $\frac{15}{2}.ks.m'/m \times 0{\cdot}229$.

Thus starting with n_o units mRNA ml^{-1} at $t = 0$, the amount present at $t = 15$ min is:

$$n_{15} = n_0 - (n_0 + \tfrac{15}{2}.ks.m'/m) \times 0{\cdot}229 + 15\ ks.m'/m. \tag{2}$$

The function of the constant k is to modify the absolute rate of mRNA synthesis so that messenger content per ml culture exactly doubles in each cell cycle, which must happen in cells undergoing balanced, exponential growth. If the messenger RNA metabolism of the culture were such that it achieved a steady state condition in every cell cycle, i.e. if the rates of mRNA synthesis and degradation became equal, the value of k would be 1. As we cannot assume that steady state is in fact reached in the *S. pombe* cell cycle, we must derive a value for k. This is done by considering an 'ideal' synchronous culture; that is, one in which mRNA synthesis is not influenced by artifacts from the synchronization procedure or loss of synchrony with time. As a basis for this ideal culture, we take the experimentally determined changes in the rate of poly(A)$^+$mRNA synthesis per ml between 2 h and 4 h 25 min (equal to 1 cell generation time) from Fig. 6, and reproduce the same curve to represent the preceding and subsequent divisions. This is shown in Fig. 10. Starting with 100 units mRNA ml^{-1}, we calculate changes in mRNA content over successive 15-min intervals using

Eq. (2). This was initially performed with $k = 1{\cdot}0$, and was repeated with different values of k until the k value was found which gave an exact doubling of mRNA content in every cell cycle. Fig. 10 shows the calculated curve for mRNA content which satisfied this condition; the k value was 1·10. This value of k implies that steady state is never reached, as at any time the rate of synthesis is always faster than the rate of degradation.

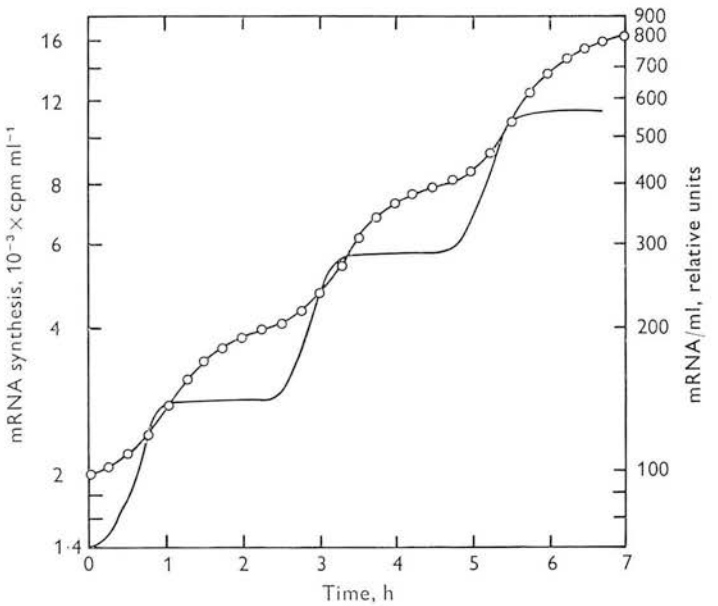

Fig. 10. Changes in relative rate of synthesis of poly(A)$^+$mRNA in an 'ideal' synchronous culture (——), and in content of mRNA per ml (○—○) calculated by Eq. (2).

We can now calculate, using Eq. (2), with $k = 1{\cdot}10$, changes in mRNA content in the real synchronous culture shown in Fig. 6. The value of m we used is the relative rate of mRNA synthesis *before* the depression of poly(A)$^+$mRNA synthesis by the synchronization procedure. This value was found by extrapolating the curve for changes in the rate of poly(A)$^+$mRNA synthesis during the second division (2 h to 4 h 25 min) back to $t = 0$ (Fig. 11). The value of m obtained is of course higher than the measured values of poly(A)$^+$mRNA synthesis rate early in culture; the difference is similar to the measured depression of poly(A)$^+$mRNA synthesis rate by the synchronization procedure shown in Fig. 2.

Fig. 11 shows the calculated curve of mRNA content in the real synchronous culture. It is clear that mRNA accumulation in the real culture is much less periodic than in the ideal culture (Fig. 10), and that the pattern of accumulation in the first cell cycle is markedly affected by the synchronization procedure. It is not until the second cycle that a periodic pattern of mRNA accumulation is established.

Calculation of a curve for protein accumulation

From the calculated curve of changes in mRNA content during synchronous culture (Fig. 11), we calculate a curve for protein accumulation. We assume that in any 15-min time interval, the amount of protein synthesized is proportional to the average mRNA content during the time interval. The protein is assumed to be stable. The amount of protein present at $t = 0$ is taken to equal the increment during the first cell-generation time in culture. Fig. 9 shows the calculated protein-accumulation data, plotted as linear and semilogarithmic plots.

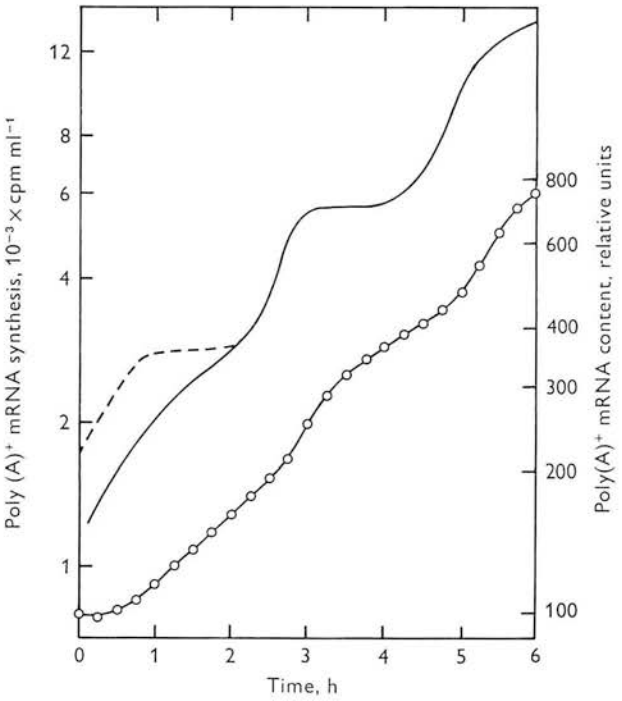

Fig. 11. Changes in messenger RNA content per ml culture in a synchronous culture (○—○), calculated using Eq. (2), p. 518, from changes in the rate of poly(A)⁺mRNA synthesis per ml (——). The poly(A)⁺mRNA synthesis data are re-drawn from Fig. 6. The broken line shows the extrapolation of the poly(A)⁺mRNA synthesis rate used to find the value of m unperturbed by the depressive effects of the synchronization procedure on poly(A)⁺mRNA synthesis.

We then tried to fit to these data curves which seemed likely patterns of protein accumulation in cultures undergoing synchronous, exponential growth. The simpler model considered was an exponential increase in protein. A single straight line was fitted to the plot of log protein content against time by the method of least squares. The second model was derived from results with bacterial systems, where some stable enzymes are known to increase in activity linearly through the cell cycle, with a doubling in the rate of accumulation once per cycle (Kuempel *et al.* 1965; Donachie & Masters, 1969). We fitted a series of straight lines by inspection to the linear plot of

the protein accumulation data, with the restriction that there could be only one rate-change point per cell cycle. The goodness of fit in each case was tested by calculating the residual sum of squares between the calculated points and the fitted line. Fig. 9 shows the best fit obtained. Three straight lines were used, with rate changes at 2h 50 min and 5 h 15 min. The increase in rate of protein accumulation at these points was close to a doubling. This segmented, linear model gave a much better fit to the calculated data than the exponential model. The residual sum of squares of the exponential fit was 5·7 times that of the best segmented linear model.

The overall doubling time of calculated protein content during the synchronous culture is the same as the cell doubling time. The 2 rate-change points in the segmented, linear model both occur at the same stage, 0·2, of the cell cycle. A possible rate change early in the first cycle was not considered, as there would be too few points to establish the initial rate.

It is clear that the calculated points for protein accumulation do not show a sharp rate change, but curve round from one linear rate to another. But DNA synthesis is not an instantaneous process; if gene replication controls the rate-doubling, the curve for total protein accumulation will be composed of many individual curves,with rate changes at slightly different times. These in total will show a smooth transition from one linear rate to the next. Imperfections of synchrony will also tend to make the rate-change gradual rather than sharp.

(Reprinted from Nature, Vol. 271, No. 5647, pp. 726–730, February 23, 1978)

Novel cell cycle control of RNA synthesis in yeast

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During the fission yeast cell cycle, the rate of polyadenylated messenger RNA synthesis doubles when the cell reaches a critical size. This size-related control maintains average mRNA content in balance with total cell mass during exponential growth, even in cells growing at different absolute growth rates per cell.

In cell cultures undergoing balanced exponential growth, individual parameters of growth remain at a constant proportion of total cell mass[1]. The two daughter cells produced at each division are identical to the parent at the same time in the preceding cycle: this requires that all cell components are doubled during the course of each cell cycle. The mechanisms controlling the balanced increase of components, and their duplication during each cell cycle are not understood.

A simple model explaining the doubling in amount of a component in each cell cycle can be based on a stepwise doubling in the rate of synthesis of that component at a fixed point in every cell cycle. In the cell cycle of the fission yeast *Schizosaccharomyces pombe* there are periodic doublings in rate of increase of total dry mass[2], rates of increase in activities of three enzymes[3], and in rates of synthesis of total RNA[4], ribosomal protein[4], ribosomal RNA[5] and polyadenylated mRNA[5]. In all cases, the doubling in rate occurs early in the cell cycle.

Two types of control may be proposed to account for the timing of the rate doublings during the cell cycle. The doublings might be dependent on DNA replication, which occurs at the beginning of the fission yeast cell cycle[6]. Alternatively, the rate doublings may be related to overall growth, being triggered when the cell reaches some critical size or mass. These two types of control mechanism may be distinguished using strains of fission yeast which have very different cell sizes[7] but in which there is relatively little variation in the timing of DNA replication in the cell cycle[7]. We have investigated the rates of synthesis of polyadenylated mRNA (polyA$^+$mRNA) during the cell cycles of cells dividing at different sizes. Our evidence suggests that the timing of the doubling in rate of polyA$^+$mRNA synthesis in the cell cycle is not controlled by the timing of DNA replication, but that doubling in the rate of mRNA synthesis is triggered when the cells reach a critical size. Such a mechanism will maintain the average ratio of polyA$^+$mRNA to total cell mass, and will enable cells of different mean sizes to double their mRNA content in each cell cycle.

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Characteristics of the wild-type and mutant strains

Table 1 shows generation times and macromolecular contents of exponentially-growing haploid cells of wild type strain 972 h$^-$ and a mutant derivative *wee* 1-50 h$^-$. The mutant strain (previously described as *cdc* 9-50 (ref. 7)) is altered in the control initiating mitosis, so that mitosis and cell division take place in cells of about half the size of wild type[7]; generation time is unaltered. Growing cells of strain *wee* 1-50 have a reduced macromolecular content per cell, with 57% of the protein, 53% of the total RNA and 59% of the polyA$^+$mRNA content of wild type. The average DNA content per cell of *wee* 1-50 is comparatively high, at 82% of wild type. This results in a ratio of polyA$^+$mRNA to DNA which is lower in *wee* 1-50 than in wild type, and therefore mRNA content per cell cannot be regulated by gene dosage alone.

Macromolecular synthesis during the cell cycle

To examine the means by which the reduced polyA$^+$mRNA content per cell of *wee* 1-50 is brought about, we measured the patterns of synthesis of DNA and polyA$^+$mRNA in synchronously-dividing cultures of *wee* 1-50 and wild type. In wild type cells, DNA replication was at the beginning of the cell cycle, almost coincident with cell division (Fig. 1). The rate of

Table 1 Characteristics of mutant and wild-type strains

	Wild type 972 h$^-$	Mutant *wee* 1-50
Generation time (min)	140	140
Cell volume at division (μm^3)	149	73
RNA (pg per cell)	3.0 ± 0.04	1.59 ± 0.07 (53%)*
Protein (pg per cell)	12.1 ± 0.3	6.85 ± 0.4 (57%)
PolyA$^+$mRNA (fg per cell)	23.8 ± 0.8	14.1 ± 0.4 (59%)
DNA (fg per cell)	34.6 ± 1.4	28.4 ± 0.8 (82%)

Cultures of the two strains were grown at 35 °C in phthalate-buffered EMM 2 medium. The length and diameter of 200 cells with cell plates were measured and cell volume calculated. Cell generation time, RNA, protein and DNA contents were determined using cultures in the early phase of exponential growth by methods described in ref. 7. PolyA$^+$mRNA content was calculated from the ultraviolet absorption spectrum of polyA$^+$mRNA isolated by affinity chromatography on oligo dT–cellulose as described in ref. 5. Polyacrylamide gel electrophoresis of the polyA$^+$mRNA fraction showed no contamination by ribosomal or transfer RNAs. Values are means ± s.e.m.

*Numbers in parentheses give the values of parameters determined in the mutant as a percentage of that determined in the wild type.

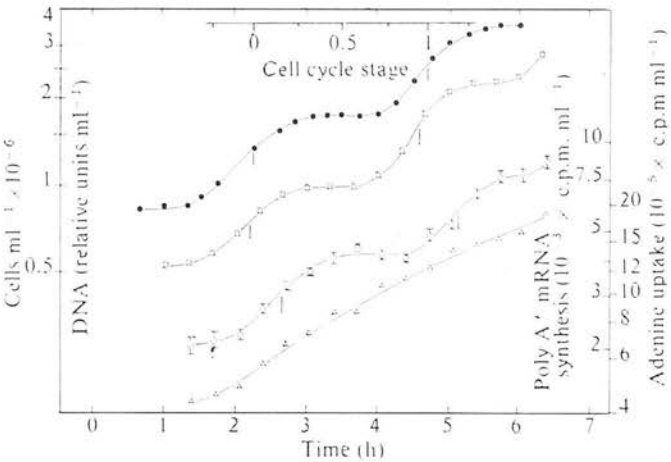

Fig. 1 Changes in cell no., DNA content and rates of adenine uptake and polyA$^+$mRNA synthesis in a synchronously-dividing culture of *Schizosaccharomyces pombe* Lindner, wild-type strain 972 h$^-$. Cells were collected by filtration from a population growing exponentially at 35 °C in phthalate-buffered EMM2 medium[7], and were fractionated by sedimentation rate centrifugation through a lactose density gradient[17] (MSE Type A zonal rotor). Small cells at the beginning of the cell cycle were selected as inoculum for the synchronous culture. Changes in cell number were monitored using a Coulter counter. Samples of 50–100 ml culture were withdrawn every 20 min and pulse-labelled by incubating for 10 min (0.07 of the cell generation time) with 2.5 μCi ml^{-1} [2-^{3}H]adenine (Radiochemical Centre). The total adenine concentration of the pulse medium was adjusted to 1 μg ml^{-1} by addition of nonradioactive adenine. Less than 10% of the radioactivity supplied was taken up during the pulse. DNA content and rates of adenine uptake and polyA$^+$mRNA synthesis were measured as described in ref. 5. Values for polyA$^+$mRNA synthesis are means ± s.e.m. of six determinations. The midpoints of the two successive doublings in cell numbers fix stages 0 and 1 on the cell cycle map. The midpoints of the stepwise doublings in DNA content and rate of polyA$^+$mRNA synthesis were taken as the time when the step attained the value midway between the two plateaus on either side of the step, and are marked by vertical bars. ●, Cell no.; □, DNA content; △, rate of adenine uptake; ○, rate of polyA$^+$mRNA synthesis.

incorporation of adenine into polyA$^+$mRNA doubled as a step, with the midpoint of the step about 0.1 to 0.2 of a cycle after the midpoint of DNA accumulation. Similar results have been obtained using ^{3}H-uridine as label instead of ^{3}H-adenine, and also with another strain of wild-type size[5].

Quite different results were obtained with synchronous cultures of *wee* 1-50. The midpoint of DNA replication was 0.2 to 0.3 of a cycle after cell division (Fig. 2), consistent with

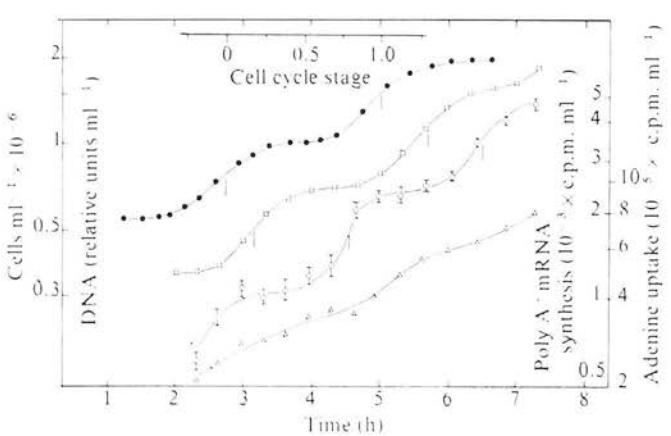

Fig. 2 Changes in cell no., DNA content and rates of adenine uptake and polyA$^+$mRNA synthesis in a synchronously-dividing culture of the mutant strain *wee* 1-50. Measurements were made and the cell cycle stage of changes derived as explained in the legend to Fig. 1. ●, Cell no.; □, DNA content; △, rate of adenine uptake; ○, rate of polyA$^+$mRNA synthesis.

previous results[7] and with the average DNA content of *wee* 1-50 cells in asynchronous culture being 82% of that of wild type (Table 1). The rate of adenine incorporation into polyA$^+$mRNA also doubled as a step, but the midpoint of the step was at an average of 0.81 of the cell cycle (Table 2). This is 0.5 of a cycle later than DNA replication in *wee* 1-50, and 0.7 of a cycle later than the corresponding step in rate of incorporation into polyA$^+$mRNA in wild type. These results suggest that the timing of the doubling in rate of incorporation into polyA$^+$mRNA is not directly controlled by the timing of DNA replication.

The pattern of increase in the rate of adenine uptake during growth of synchronous cultures is rather variable (Figs 1 and 2, and ref. 5). However, the stepwise increases in the rate of adenine incorporation into polyA$^+$mRNA were greater than any periodic changes in adenine uptake. This suggests that the changes in rate of adenine incorporation into polyA$^+$mRNA resulted from changes in rate of synthesis and not from changes in rate of precursor uptake. Control experiments were carried out to check whether the doublings in rate of polyA$^+$mRNA synthesis were merely periodic fluctuations induced by the physiological trauma of synchronous culture preparation. Cells from the parent culture were exposed to all the conditions used to prepare synchronous cultures, but the inoculum selected contained cells at all stages of the cell cycle. The resulting culture showed an exponential, asynchronous increase in cell number. The rate of adenine incorporation into polyA$^+$mRNA also increased continuously with time: there was no suggestion of the steps in rate of incorporation seen in synchronous culture. We conclude that the stepwise doublings in rate of polyA$^+$mRNA synthesis observed in synchronous cultures were cell cycle-related events.

Table 2 PolyA$^+$mRNA synthesis during the cell cycles of wild type and *wee* 1–50 mutant strains

	Wild type 972 h$^-$	Mutant *wee* 1-50
Cell cycle stage of midpoint of stepwise doubling in rate of polyA$^+$mRNA synthesis	0.11 ± 0.04	0.81 ± 0.06
Rate of polyA$^+$mRNA synthesis per cell before stepwise rate-doubling (10^3 × c.p.m.)	1.24 ± 0.05	1.29 ± 0.03
Rate of polyA$^+$mRNA synthesis per cell after stepwise rate-doubling (10^3 × c.p.m.)	2.43 ± 0.15	2.39 ± 0.14

Data were derived from synchronous culture experiments such as shown in Figs 1 and 2. The time of the midpoint of stepwise doubling in rate of polyA$^+$mRNA synthesis was expressed as stage in the cell cycle as shown in Fig. 1. Rates of polyA$^+$mRNA synthesis per cell before and after the stepwise doubling in rate were calculated by dividing the steady rates of incorporation before and after the step by the cell number per ml after separation of the daughter cells formed at the beginning of that cell cycle. Values are means ± s.e.m.

Dependency on DNA replication

Although in the cell cycle of *wee* 1-50 the timing of the doubling in rate of synthesis of polyA$^+$mRNA is not closely linked with the timing of DNA replication, the doubling in rate of polyA$^+$mRNA synthesis might still depend on completion of the previous round of DNA replication. We examined this possibility by following the rate of synthesis of polyA$^+$mRNA in an asynchronous, exponentially-growing culture of *wee* 1-50 after inhibition of DNA synthesis by hydroxyurea. The rate of DNA synthesis fell to about one quarter of its initial level within 5 min of addition of the inhibitor (Fig. 3). The rate of synthesis of polyA$^+$mRNA continued to increase at approximately the

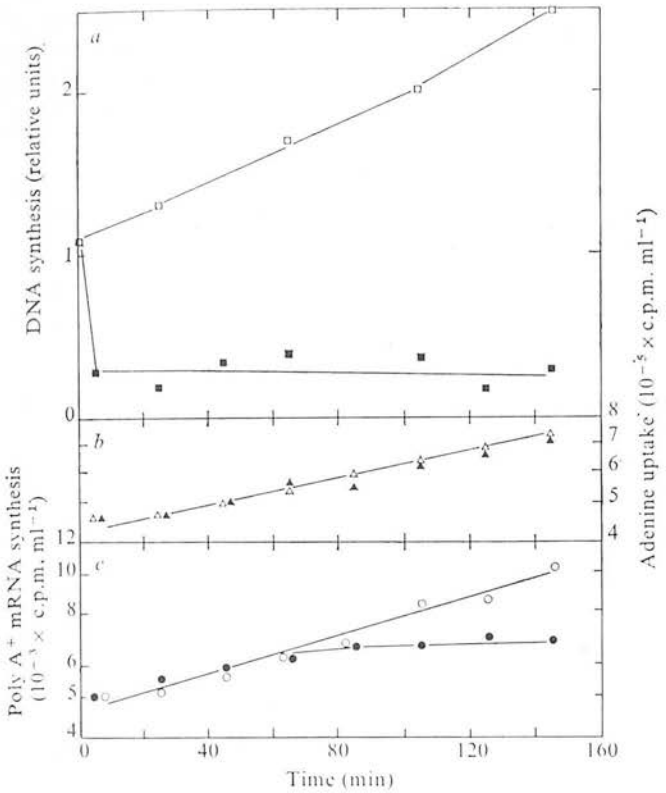

Fig. 3 Changes in rates of DNA synthesis, adenine uptake and polyA$^+$mRNA synthesis in a culture of *wee* 1-50 after inhibition of DNA synthesis. An exponentially-growing culture containing 10^6 cells ml^{-1} was split in two. Hydroxyurea (11 mM) was added to one half at 0 min to inhibit DNA synthesis. The other half served as control. The rate of DNA synthesis was measured as described in ref. 18. Rates of adenine uptake and polyA$^+$mRNA synthesis were measured as described in the legend to Fig. 1. *a*, DNA synthesis; *b*, rate of adenine uptake; *c*, rate of polyA$^+$mRNA synthesis. Open symbols represent values for the control culture, closed symbols for the hydroxyurea-treated culture.

same rate as in the control for about 70 min, then the rate became constant. This 70-min delay is similar to the period between DNA replication and doubling in rate of polyA$^+$mRNA synthesis in synchronously-dividing cultures of *wee* 1-50 (Fig. 2). These results fit the following model: the doubling in rate of synthesis of polyA$^+$mRNA is dependent on the occurrence of the preceding round of DNA replication. After DNA replication, a cell is potentially capable of doubling its rate of polyA$^+$mRNA synthesis, but in *wee* 1-50 does not do so until 0.5 of a cycle later (Fig. 2, Table 2). In an asynchronous population, there will be a fraction of cells which have completed DNA replication but which have yet to double their rate of polyA$^+$mRNA synthesis. These cells are responsible for the 70-min period during which the rate of polyA$^+$mRNA synthesis rose after inhibition of DNA synthesis (Fig. 3). Consistent with this interpretation, in cells of wild type size, where doubling in rate of polyA$^+$mRNA synthesis follows closely on DNA replication (Fig. 1, Table 2), hydroxyurea added to an asynchronous culture causes the rate of polyA$^+$mRNA synthesis to become constant within 10 to 20 min (ref. 5).

Hydroxyurea is the only inhibitor of yeast DNA synthesis known to give the very rapid inhibition required for these experiments[8]. Its use is open to the criticism that hydroxyurea reportedly inhibits precursor uptake and growth (ref. 8 and J. M. Mitchison and J. Creanor, unpublished). Adenine uptake was not inhibited in our experiments (Fig. 3), but we cannot entirely exclude the possibility that hydroxyurea was having some unknown side effect which prevented further rise in the rate of polyA$^+$mRNA synthesis.

Explanation for the reduced polyA$^+$mRNA content of *wee* 1-50

We have shown that the rate of polyA$^+$mRNA synthesis doubles much later in the cell cycle of *wee* 1-50 than in wild type. This difference can be shown to be sufficient to account for the reduced polyA$^+$mRNA content of the average *wee* 1-50 cell in exponential culture (Table 1), by calculating the polyA$^+$mRNA content of *wee* 1-50 if the delay in doubling were the only difference between the two strains. The absolute rates of polyA$^+$mRNA synthesis per cell before and after the step doubling in rate are the same for *wee* 1-50 and wild type (Table 2). We assume that the half life of the polyA$^+$mRNA is the same in both strains. In these conditions, the mean polyA$^+$mRNA content of *wee* 1-50 cells in asynchronous, exponential growth is 2^{-l} of the wild-type content, where l is the delay, as a fraction of the cell cycle, between doubling in rate of polyA$^+$mRNA synthesis in the two strains. The derivation of this relationship is to be described in detail elsewhere (A. Barnes, P.N. and R.S.S.F., in preparation). The average delay between doubling of the rate of polyA$^+$mRNA synthesis in wild type and *wee* 1-50 was 0.70 of the cell cycle (Table 2). This gives a calculated value for mean polyA$^+$mRNA content of *wee* 1-50 cells of 62% of the wild-type content. This is in good agreement with the observed value of 59% (Table 1), and indicates that the delay in doubling the rate of polyA$^+$mRNA synthesis in *wee* 1-50 cells is sufficient to explain their reduced polyA$^+$mRNA content.

Cell size control over polyA$^+$mRNA synthesis

We now consider the nature of the mechanism which determines that the rate of polyA$^+$mRNA synthesis doubles early in the cell cycle of wild type and late in the cycle of *wee* 1-50. *Wee* 1-50 cells at the end of their cycle are similar in size to wild-type cells at the beginning of their cycle, suggesting that some aspect of

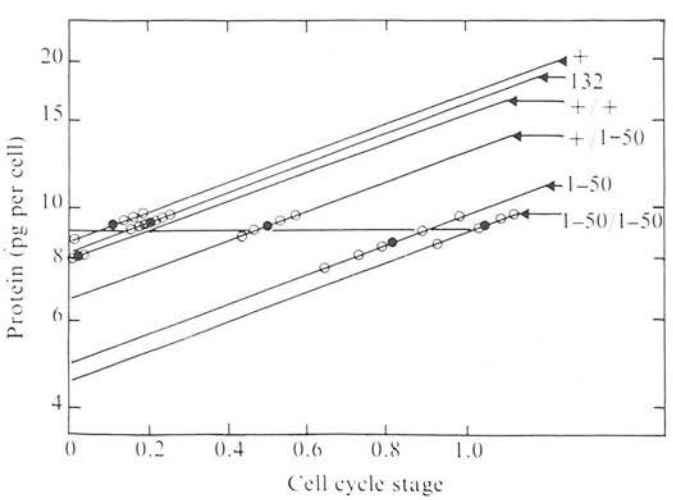

Fig. 4 Relationship between cell protein content and cell-cycle time of the stepwise doubling in rate of polyA$^+$mRNA synthesis, in yeast strains of a wide range of sizes. Each open circle represents the midpoint of a stepwise doubling in rate of polyA$^+$mRNA synthesis, measured in synchronous cultures as shown in Fig. 1. Closed circles represent the mean values for each strain. The yeast strains used were 972 wild-type haploid (+); strain 132 haploid (132); 972 diploid wild type (+/+); *wee* 1-50 haploid (1-50) *wee* 1-50 diploid (1-50/1-50) and the heterozygous diploid of *wee* 1-50 and 972 wild type (+/1-50). The cell-cycle time scale is the time between the midpoints of two successive divisions. The points for time of doubling in rate of polyA$^+$mRNA synthesis are plotted along a line showing the increase in protein content during the cell cycle for each strain. These lines were calculated from the mean protein content per cell in asynchronous, exponential culture for each of the strains knowing that protein content per cell increases close to exponentially during the cell cycle[9]. The protein per cell data for the three diploid lines have been divided by two to permit comparison with the haploid lines.

cell size might be important. We have studied this quantitatively by taking cell protein content as a measure of size. Knowing the protein content per cell in asynchronous, exponential culture (Table 1), and that protein per cell increases close to exponentially through the cell cycle[9], we can calculate changes in protein per cell during the cell cycle of each strain. Figure 4 shows that in *wee* 1-50 and wild type, the doubling in rate of polyA$^+$mRNA synthesis occurred in cells with similar protein contents. To examine this further, we have measured the time when the rate of polyA$^+$mRNA synthesis doubles in another haploid strain (132) of wild-type size[5], and in a series of diploid strains of different sizes. All strains investigated showed a stepwise doubling in the rate of polyA$^+$mRNA synthesis in synchronous culture; all have similar mean generation times. The protein contents of the diploid strains have been divided by two before plotting (Fig. 4), to allow comparison directly with the haploid strains.

In the six strains, the times at which the rate of polyA$^+$mRNA synthesis doubles are distributed throughout the cell cycle. However, all strains double their rates of polyA$^+$mRNA synthesis when the cells have very similar levels of protein per haploid genome (Fig. 4). This suggests a cell size-related control over the timing of the doubling in rate of polyA$^+$mRNA synthesis in the cell cycle.

We propose that in cells in steady-state growth, the rate of synthesis of the majority of polyadenylated mRNAs is under a general control. This sets the rate per cell at one of two levels, the higher rate being double the lower. The cell switches from the lower to the higher rate on attaining a critical size or mass. The control consists of two parts: the recognition of the threshold size, and the means by which the rate of polyA$^+$mRNA synthesis is maintained at the two discrete levels.

Recognition of the threshold cell size might involve monitoring some aspect of cell growth. Such size-monitoring mechanisms have been proposed as controls of initiation of DNA replication and mitosis[10]. An example would be the 'inhibitor dilution' model as proposed by Pritchard[11]. A regulator of mRNA synthesis would be synthesised as a pulse once per cycle. When the concentration of this regulator is reduced to a threshold value by increase in cell volume, the doubling in rate of polyA$^+$mRNA synthesis would be effected. Our comparison of haploid and diploid strains suggests that the amount of regulator synthesised would be a constant amount per haploid genome, not a constant amount per cell. We do not know the nature of this regulator, but a promising candidate would be a molecule analagous to 'magic spot' which is involved in a stringent type of control[12].

The simplest mechanism whereby the rate of mRNA synthesis could be maintained at one rate or twice that rate is one based on a modification of the gene dosage model. This type of model is supported by our experiments on the rate of polyA$^+$mRNA synthesis after inhibition of DNA accumulation by hydroxyurea (Fig. 3), and by the observation that the absolute rates of polyA$^+$mRNA synthesis per cell before and after the step doubling are the same in *wee* 1-50 and wild type (Table 2). However, since the doubling in rate of polyA$^+$mRNA synthesis may occur much later than DNA replication, as in *wee* 1-50, there must be some further mechanism to maintain the rate of polyA$^+$mRNA synthesis at the pre-replication rate until the threshold cell size is reached. This could involve some form of temporary masking of one of the two copies of each gene present after replication[3,13-15], only one of which would be active until the cell had attained the threshold size. Alternatively, there could be some form of gene dosage compensation such as is found in Drosophila[16]. In this model the rate of transcription of each of the two gene copies present after DNA replication would be half that of the single copy present before replication. The rate of transcription of both copies would be doubled when the cell attained the threshold size. This control could act through the availability of RNA polymerase or its regulatory subunits, though in these cases it is not clear why the rate of transcription should necessarily double as a step.

The proposed general control over rate of mRNA synthesis does not exclude the possibility that the rate of transcription of individual genes is also subject to other, more specific controls.

Significance of cell size control

The cell size-related control over time of doubling in rate of polyA$^+$mRNA synthesis has several implications for the control of balanced exponential growth. It will maintain the average polyA$^+$mRNA content per cell as a constant proportion of total cell mass during exponential growth, and will ensure that polyA$^+$mRNA content per cell doubles over the course of each cell cycle. This regulation of balanced growth and duplication will operate even in cells dividing at different sizes and growing at different absolute growth rates per cell. Thus the control could maintain the concentration of polyA$^+$mRNA within a range suitable for further post-transcriptional modulation.

It has been shown[5] that a doubling in the rate of mRNA synthesis in each cell cycle could lead to observed patterns of accumulation and doubling of certain enzymes in each cell cycle[3]. A cell size-related control over the time of doubling in rate of mRNA synthesis therefore offers the possibility of a widespread regulation of the synthesis of those enzymes not subject to specific controls at the transcriptional or translational levels.

We thank Laurence Errington and Robert Pitcairn for technical assistance and Tahia Benitez and Murdoch Mitchison for constructive criticisms. The MRC and SRC provided financial support.

Received 24 October 1977; accepted 6 January 1978.

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Printed in Great Britain by Henry Ling Ltd., at the Dorset Press, Dorchester, Dorset

J. Cell Sci. **35**, 25–40 (1979)
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ALTERED PATTERNS OF RIBONUCLEIC ACID SYNTHESIS DURING THE CELL CYCLE: A MECHANISM COMPENSATING FOR VARIATION IN GENE CONCENTRATION

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SUMMARY

In the fission yeast *Schizosaccharomyces pombe*, a series of diploid mutants divides at smaller cell sizes than wild type. In these smaller strains, the mean gene concentration (defined by previous authors as the DNA to protein ratio) is higher than in wild type. Such an increase in gene concentration should also increase the concentration of those components such as messenger and ribosomal RNA, whose rate of synthesis is determined by gene dosage. We show that the mean concentrations of these 2 RNA species in the small cells are not increased, but are the same as in wild type. The small mutant cells are thus able to compensate for changes in gene concentration. This compensation is shown to operate through differences in the patterns of synthesis of RNA during the cell cycle. In all the strains of the diploid series, the rates of synthesis of messenger and ribosomal RNA double as steps once in each cell cycle. The timings of the steps in the cell cycle appear to be cell-size related, since the smaller the cell at division, the later are the steps in the cell cycle. In contrast, there is comparatively little variation in the timing of DNA replication in the cycles of cells of different sizes. We propose that after DNA replication, there is a delay before doubling in the rate of transcription. Such a cell mass-related delay is all that is required to compensate for increased gene concentration, and results in the same mean functional DNA concentration in all strains. This mechanism will maintain the same mean messenger and ribosomal RNA concentrations in cells dividing at different sizes. Ways in which the cell size-related control over transcription may operate are discussed.

INTRODUCTION

Gene concentration can vary between cells of the same species. There are 3 ways in which this variation can arise. The mean number of copies of a gene per cell can vary, an example being the genes carried on the X chromosome. In a cell of the homogametic sex (usually the male) with one X chromosome, there are half as many copies of these genes as in a cell of the heterogametic sex (usually the female) with 2 X chromosomes. Secondly, gene concentration will be altered if the mean number of gene copies per cell remains the same, but if mean cell size varies. Thus a cell smaller than normal will have a higher mean gene concentration. Thirdly, any alteration in the timing of DNA replication during the cell cycle will alter the average gene concentration of cells in steady-state growth.

Variation in gene concentration poses questions about the regulation of synthesis of

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gene products. For genes regulated autogenously or by feedback effectors, variation in gene product concentration will be less than corresponding variation in gene concentration, and may become negligible if the power of control in the regulatory circuit is sufficiently high (Chandler & Pritchard, 1975). A contrasting case is where the rate of synthesis of a gene product is determined by gene dosage, that is by the number of copies of the gene in the cell. In both prokaryotes and eukaryotes, there is evidence that gene dosage can be a major component of the controls limiting rates of synthesis of certain proteins (Hilger *et al.* 1973; Reichart & Winter, 1975; Kuempel, Masters & Pardee, 1965; Donachie, 1965; Helmstetter, 1968) and of messenger and ribosomal RNAs (Pfeiffer & Tolmach, 1968; Klevecz & Stubblefield, 1967; Fraser & Carter, 1976; Fraser & Moreno, 1976). In the absence of other levels of control, the mean concentration of those gene products regulated at the gene dosage level will reflect variation in gene concentration. It may be difficult for the cell to accommodate such changes in concentration of gene products and to achieve balanced, steady-state growth, particularly if the activity of a significant number of genes is regulated solely at the gene dosage level.

In this paper we explore the consequences of varying gene concentration in the simple eukaryote *Schizosaccharomyces pombe*. In this organism the rates of both ribosomal and polyadenylated messenger RNA synthesis double as steps just after DNA replication in each cell cycle (Wain & Staatz, 1973; Fraser & Moreno, 1976). The experimental evidence suggests that the rates of RNA synthesis may be determined by gene dosage (Fraser & Moreno, 1976; Fraser & Nurse, 1978). Gene concentration can be conveniently altered in *S. pombe* by making use of a mutation in the gene *wee* 1 which determines the cell size at which nuclear division is initiated. Haploid cells of a mutant strain *wee* 1–50 divide at approximately half the size of haploid wild type cells (Nurse, 1975). We have used diploid lines derived from these haploid parents. The diploid *wee* 1–50 strain divides at about half the size of the diploid wild type, while the heterozygous *wee* 1–50/wild type diploid is intermediate in cell size at division (P. Nurse & P. Thuriaux, unpublished results).

We have studied the control of RNA synthesis in these 3 strains, to find how they accommodate their different gene concentrations. Our results suggest that in cells dividing at a smaller mean size than wild type, the step doublings in rates of RNA synthesis occur much later in the cell cycle than DNA replication. The point at which the step doubling occurs seems to be cell mass related, since the smaller the mean cell size, the greater the delay. We suggest that this phenomenon may compensate for the observed variation in gene concentration.

A brief description of some of our conclusions from experiments with haploid and diploid strains has been published (Fraser & Nurse, 1978). The purpose of the present paper is to present full experimental results for the diploid strains, and discuss further aspects of the control mechanism. A theoretical analysis of the consequences of a size control over transcription for the control of balanced exponential growth is given in the following paper (Barnes, Nurse & Fraser, 1978).

MATERIALS AND METHODS

Strains and genetical procedures

We used the standard genetical procedures for *Schizosaccharomyces pombe* Lindner as described by Gutz, Heslot, Leupold & Loprieno (1974). Derivation of strain *wee* 1–50 from wild type strain 972 h^- was as described by Nurse (1975). Diploid strains were constructed as described by Nurse, Thuriaux & Nasmyth (1976) using the mating-type allele *mei* 1–102 as described by Egel (1973).

Culture conditions

Cultures were grown with stirring at 35 °C in a minimal medium EMM2 (Mitchison, 1970) as modified by Nurse (1975). Synchronous cultures of 1000 to 1300 ml with a starting density of $1–2 \times 10^6$ cells/ml were prepared by selection of small cells from 15 l. of exponentially growing asynchronous culture with a density no greater than 5×10^6 cells/ml. Selection of small cells was by sedimentation through a 7·5 to 30 % gradient of lactose in culture medium using a M.S.E. Type A zonal rotor (Mitchison & Carter, 1975). Changes in cell number were monitored using a Coulter Counter as described by Mitchison (1970).

Radioactive labelling

Samples of 50 to 80 ml synchronous culture, containing $1–2 \times 10^8$ cells, were withdrawn at 20-min intervals. These samples were pulse-labelled for 10 min with 2·5 μCi/ml [2-^{3}H]adenine (Radiochemical Centre, Amersham, U.K.). The total adenine concentration was adjusted to 1 or 3 μg/ml with non-radioactive adenine. Under these conditions, less than 10 % of the isotope supplied was taken up during the labelling period. At the end of the pulse, crushed ice was added to stop further incorporation. Cells were harvested by filtration on an Oxoid membrane filter with 0·45-μm pore diameter. Adenine uptake by the cells was measured and total nucleic acid was extracted by a detergent-phenol procedure (Fraser & Moreno, 1976). The rate of synthesis of ribosomal RNA (rRNA) was determined from the radioactivity of the 25 S plus 18 S rRNA peaks after fractionation of samples of total nucleic acid on polyacrylamide gels (Loening, 1967) as explained by Fraser & Carter (1976). The rate of synthesis of polyadenylated messenger RNA (poly(A)$^+$mRNA) was measured by the radioactivity of that fraction of total RNA binding to oligo(deoxythymidylic acid)-cellulose at high salt concentration. Details of the assay and characteristics of the poly(A)$^+$mRNA fraction isolated are given elsewhere (Fraser, 1975). Changes in DNA content during synchronous culture were determined as described by Fraser & Moreno (1976).

To measure the rate of total RNA synthesis in asynchronous, exponentially-growing cultures, 1-ml samples containing $1–2 \times 10^6$ cells were incubated with 2·5 μCi/ml [2-^{3}H]adenine at a total adenine concentration of 1 μg/ml for 5 min. Incorporation of adenine into cold-acid-insoluble material was measured as explained by Fraser & Moreno (1976).

Mean DNA, protein and total RNA contents of cells in asynchronous, exponential culture were measured as described by Nurse (1975) and Nurse & Thuriaux (1977). Mean poly(A)$^+$mRNA content was calculated from the ultraviolet absorption spectrum of the poly(A)$^+$mRNA fraction isolated as above.

Asynchronous control cultures

Cells harvested from an asynchronous exponential culture were exposed to conditions similar to those used for preparation of synchronous cultures. The cells were resuspended in medium: the suspension was slowly diluted, and the lactose concentration increased, to mimic the zonal centrifugation treatment used to prepare synchronous cultures. The final lactose concentration to which control cells were exposed equalled that in the region of the zonal rotor from which the inoculum for a synchronous culture was taken. The total time of exposure to high cell density and lactose was similar to the time required for a zonal rotor fractionation of cells. A sample of the lactose-treated cells, containing cells of all sizes and hence of all stages in the cell cycle, was used as inoculum for the asynchronous control culture.

Table 1. *Generation times, volume at division and mean protein and DNA contents of 3 diploid strains of Schizosaccharomyces pombe*

Strain	Mean generation time, min	Mean volume at division, μm^3	Protein, pg/cell	DNA, fg/cell
Wild type/wild type	140	283	22·6	62·8
wee 1–50/wild type	160	231	18·5	60·2
wee 1–50/*wee* 1–50	205	144	13·0	50·1

Mean generation time, DNA and protein contents were measured in asynchronous cultures in the early phase of exponential growth. Mean cell volume at division was measured on samples of 200 cells with cell plates as described by Nurse (1975).

Table 2. *Concentrations (expressed as μg per μg total protein) of DNA, total RNA and poly(A)⁺mRNA in 3 diploid strains of S. pombe*

Strain	DNA/ protein	Total RNA/ protein	Poly(A)⁺mRNA/ protein
Wild type/wild type	$2{\cdot}78 \times 10^{-3}$	0·219	$0{\cdot}72 \times 10^{-3}$
wee 1–50/wild type	$3{\cdot}25 \times 10^{-3}$	0·223	$0{\cdot}72 \times 10^{-3}$
wee 1–50/*wee* 1–50	$3{\cdot}85 \times 10^{-3}$	0·225	$0{\cdot}72 \times 10^{-3}$

RESULTS

Characteristics of the yeast strains

Table 1 shows that at division, *wee* 1–50 diploid cells are approximately half the volume of wild type diploid cells, while the heterozygous diploid is intermediate between its 2 homozygous parents. As *S. pombe* cells are slightly irregular in shape, cell volume is difficult to measure accurately, especially if changes in volume through the cell cycle are to be considered. We will therefore use total protein per cell as a more reliable and easily measurable index of cell size. Table 1 shows that by this criterion also, *wee* 1–50 diploid cells are about half the size of wild type diploid cells, with the heterozygous diploid again intermediate between its 2 parents. In exponentially growing, asynchronous cultures, the mean generation time of the heterozygous diploid is close to that of wild type diploid cells. The mean generation time of *wee* 1–50 diploid cells is appreciably longer than that of wild type diploids. This difference will be discussed in more detail later.

Using total protein content as a measure of cell size, we have calculated mean cell concentrations (i.e. per unit total protein) of DNA, poly(A)⁺mRNA and total RNA (Table 2). It is clear that there is considerable variation in gene concentration (DNA to protein ratio) between the 3 strains. In contrast, poly(A)⁺mRNA and total RNA concentrations are identical in all 3 strains. Thus cells are able to compensate for variation in gene concentration. To investigate further the mechanisms maintaining poly-(A)⁺mRNA and total RNA concentrations at the same levels in all strains, we followed the patterns of RNA synthesis during the cell cycle for each strain.

Patterns of macromolecular synthesis through the cell cycle

Fig. 1 shows results obtained from a synchronously dividing culture of wild type diploid cells. Cell number per ml increased in a series of steps. The mid-points of 2 successive step-doublings in number are taken to mark 0·0 and 1·0 of the cell cycle. The time during the cell cycle at which a particular event occurs is expressed as a fraction of the cell cycle using this scale.

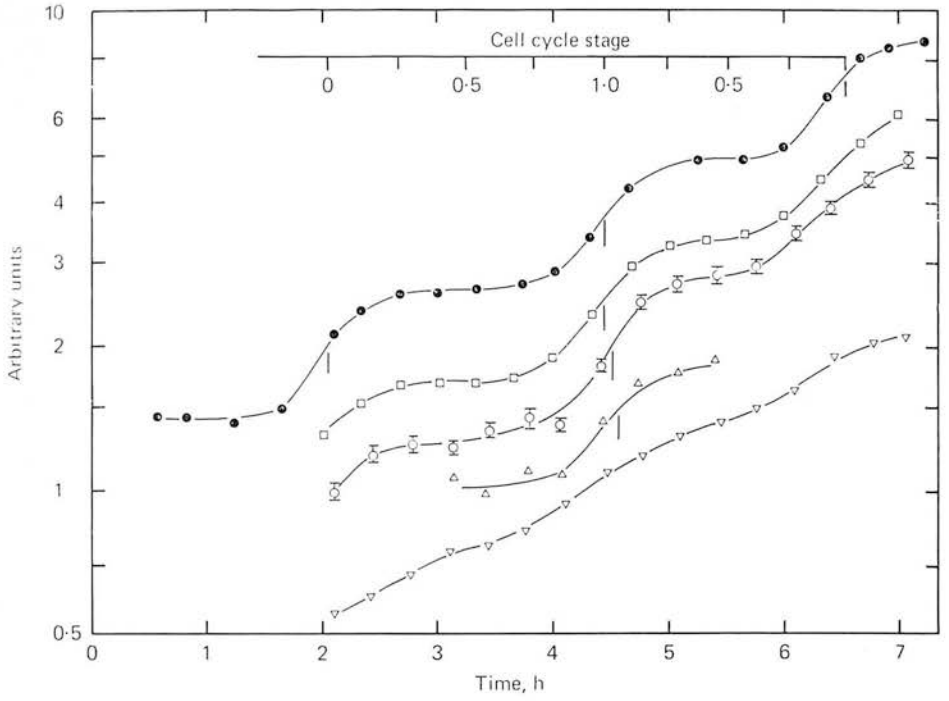

Fig. 1. Changes during growth of a synchronous culture of wild type/wild type diploid cells of *Schizosaccharomyces pombe*. The vertical scale is a log scale marked in arbitrary units; the absolute value of 1 scale unit is indicated in brackets for each parameter. ●, cells/ml (1 scale unit equals 10^6 cells/ml); □, DNA content per ml culture (relative units); ○, rate of poly(A)$^+$mRNA synthesis per ml culture (1 scale unit equals 2500 cpm/ml culture); △, rate of ribosomal RNA synthesis per ml culture (relative units); ▽, rate of adenine uptake per ml culture (1 scale unit equals 2×10^5 cpm/ml). Values for poly(A)$^+$mRNA synthesis are means ± standard errors of 6 determinations. The vertical bars on each curve show the mid-point of a stepwise increase in that parameter, measured as half-way between the plateaux levels before and after the step.

In wild type diploid cells, DNA replication occurred very early in the cycle, at about the same time as cell division (Fig. 1). A similar early timing of DNA replication has been reported for haploid wild type cells (Mitchison & Creanor, 1971). The rate of poly(A)$^+$mRNA synthesis rose discontinuously during growth of the culture, with a stepwise doubling in rate early in each cell cycle. The mid-point of the doubling was 0·0 to 0·1 of a cycle later than the mid-point of DNA accumulation. Although fewer

observations were made, the rate of synthesis of rRNA also appeared to double in a stepwise manner during the cell cycle, at about the same time as the doubling in rate of poly(A)$^+$mRNA synthesis. Similar early doublings in the rates of rRNA and poly-(A)$^+$mRNA synthesis have been reported in the cell cycle of haploid cells of wild type size (Fraser & Moreno, 1976; Fraser & Nurse, 1978).

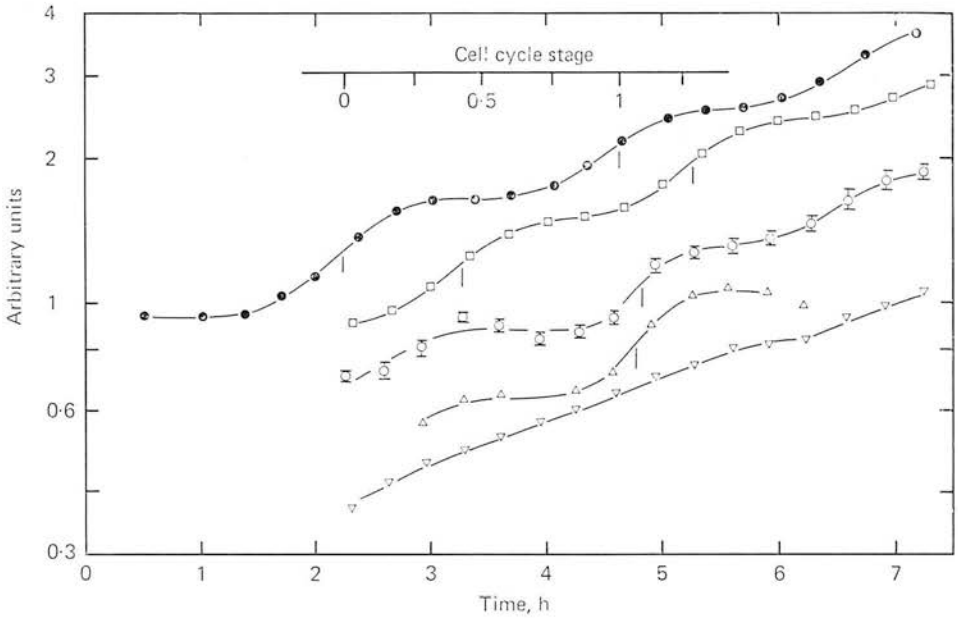

Fig. 2. Changes during growth of a synchronous culture of *wee* 1–50/*wee* 1–50 diploid cells of *S. pombe*. The vertical scale is a log scale marked in arbitrary units: the absolute value of 1 scale unit is indicated in brackets for each parameter. ●, cells/ml (1 scale unit equals 10^6 cells/ml); □, DNA content per ml culture (relative units); ○, rate of poly(A)$^+$mRNA synthesis per ml culture (1 scale unit equals 1250 cpm/ml); △, rate of ribosomal RNA synthesis per ml culture (relative units); ▽, rate of adenine uptake per ml culture (1 scale unit equals 2×10^5 cpm/ml). Values for poly(A)$^+$mRNA synthesis are means ± standard errors of 6 determinations. The vertical bars on each curve show the mid-point in a stepwise doubling in that parameter.

Fig. 2 shows that in a synchronous culture of the homozygous *wee* 1–50 diploid, the mid-point of DNA replication was at 0·3 to 0·4 of the cycle. This is later than in wild type diploid cells (Fig. 1) and a little later than in haploid *wee* 1–50 cells (Nurse, 1975). The late occurrence of DNA replication in the *wee* 1–50 diploid is consistent with its mean DNA content per cell in asynchronous, exponential culture being lower than that of wild type diploid cells (Table 1). The rates of poly(A)$^+$mRNA and rRNA synthesis in synchronous cultures of *wee* 1–50 diploid cells increased periodically, with stepwise increases in rate once per cell cycle (Fig. 2). However, in contrast to wild type, the mid-points of the steps were more than half a cycle later than the mid-point of DNA replication, and were close to the end of the cycle. The stepwise increases in rates of RNA synthesis in homozygous *wee* 1–50 diploid cells were not doublings, but

were between 1·5 and 1·7 times. Similarly, at each division the increase in cell numbers was within the range 1·5 to 1·8 times. This lack of doublings will be discussed later.

In synchronous cultures of the heterozygous *wee* 1–50/wild type diploid (Fig. 3) the mid-point of DNA replication was at the start of the cycle, as in wild type diploid cells (Fig. 1). Poly(A)$^+$mRNA and rRNA synthesis each showed a single stepwise doubling in rate during each cell cycle. The mid-points of the rate doublings occurred at about mid-cycle, considerably later than the corresponding rate doublings in wild type cells, and about half a cycle later than the mid-point of DNA replication in the heterozygous diploid.

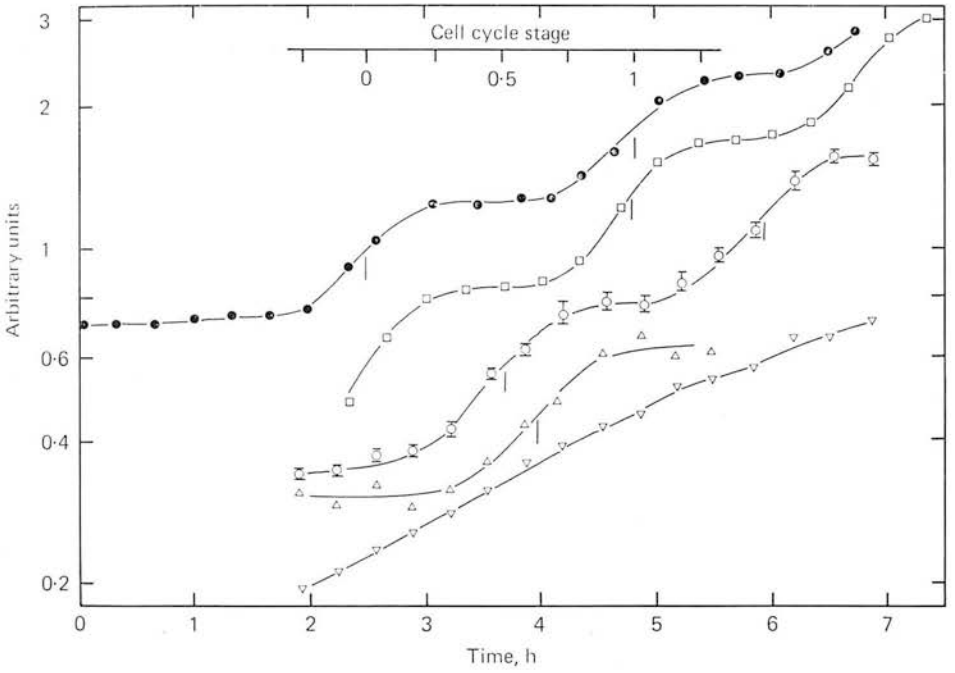

Fig. 3. Changes during growth of a synchronous culture of *wee* 1–50/wild type heterozygous diploid cells of *S. pombe*. The vertical scale is a log scale marked in arbitrary units; the absolute value of 1 scale unit is indicated in brackets for each parameter. ●, cells/ml culture (1 scale unit equals 10^6 cells/ml); □, DNA content per ml culture (relative units); ○, rate of poly(A)$^+$mRNA synthesis per ml culture (1 scale unit equals 2500 cpm/ml); △, rate of ribosomal RNA synthesis per ml culture (relative units); ▽, rate of adenine uptake per ml culture (1 scale unit equals 3×10^5 cpm/ml). Values for rate of poly(A)$^+$mRNA synthesis are means ± standard errors of 6 determinations. The vertical bars on each curve show the mid-point of a stepwise doubling in that parameter.

We have interpreted changes in the rate of incorporation of adenine into RNA as reflecting changes in the rate of RNA synthesis, rather than merely changes in the rate of adenine uptake by the cells. Figs. 1–3 show changes in the rate of adenine uptake during synchronous cultures of the 3 strains. In each case, the rate of adenine uptake increased continuously, or close to continuously. Therefore changes in the rate of adenine uptake are unlikely to have accounted for the sharp changes in rate of incorporation of adenine into RNA.

Before considering the stepwise changes in rates of rRNA and poly(A)$^+$mRNA synthesis as cell cycle-related events, it is necessary to establish that they are not merely artifacts produced by the conditions required to prepare a synchronously dividing culture. In control experiments, changes in the rate of poly(A)$^+$mRNA and rRNA synthesis were examined in cultures inoculated with cells which had been exposed to the conditions used for preparation of a synchronous culture, but in which the inoculum consisted of cells from all stages of the cell cycle. Fig. 4 shows an

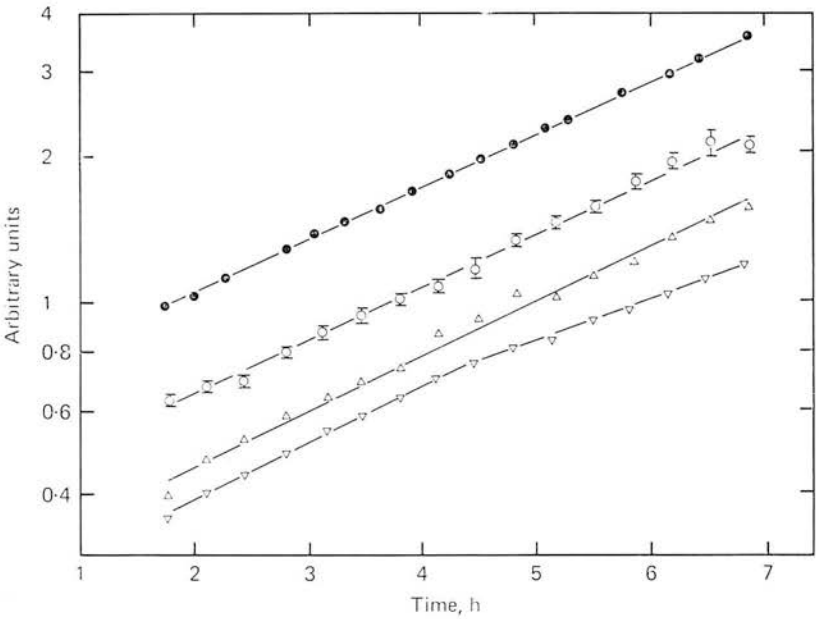

Fig. 4. Changes during growth of an asynchronous control culture of *S. pombe*. The vertical scale is a log scale marked in arbitrary units. The absolute value of 1 scale unit is indicated in brackets for each parameter. ●, cells/ml (1 scale unit equals 10^6 cells/ml); ○, rate of poly(A)$^+$mRNA synthesis per ml culture (1 scale unit equals 3000 cpm/ml); △, rate of ribosomal RNA synthesis per ml culture (relative units); ▽, rate of adenine uptake per ml culture (1 scale unit equals 3×10^5 cpm/ml). Values for rate of poly(A)$^+$mRNA synthesis are means ± standard errors of 6 determinations.

example of a control culture. Cell number, and rates of adenine uptake, poly(A)$^+$mRNA synthesis and rRNA synthesis all rose continuously, with no suggestion of discontinuous changes in rate. We conclude that the stepwise changes in rates of RNA synthesis found in synchronously dividing cultures are cell cycle-related events.

Relationship between DNA replication and changes in rate of RNA synthesis

Earlier experiments with haploid cells of wild type size (Fraser & Moreno, 1976) suggested that the stepwise doubling in rate of poly(A)$^+$mRNA synthesis depended on the occurrence of the immediately preceding round of DNA replication. In the case of the heterozygous *wee* 1–50/wild type diploid and homozygous *wee* 1–50 diploids, there was a long delay between DNA replication and the doubling in rate of RNA synthesis

in synchronous culture. To test whether these late changes in rate of RNA synthesis depended on the preceding round of DNA replication, we followed the rate of total RNA synthesis in asynchronous cultures after inhibition of DNA synthesis by hydroxyurea. At a concentration of 11 mM, this inhibitor depresses DNA synthesis to less than one quarter of the control rate within 5 min of addition (Mitchison & Creanor, 1971; Fraser & Nurse, 1978). Fig. 5 shows that in wild type diploid cells, the rate of RNA synthesis became constant within 10 to 15 min of addition of the inhibitor. In homozygous *wee* 1–50 diploid cells, the delay between addition of inhibitor and onset of a constant rate of RNA synthesis was 80–100 min, or about 0·6 of a cell cycle.

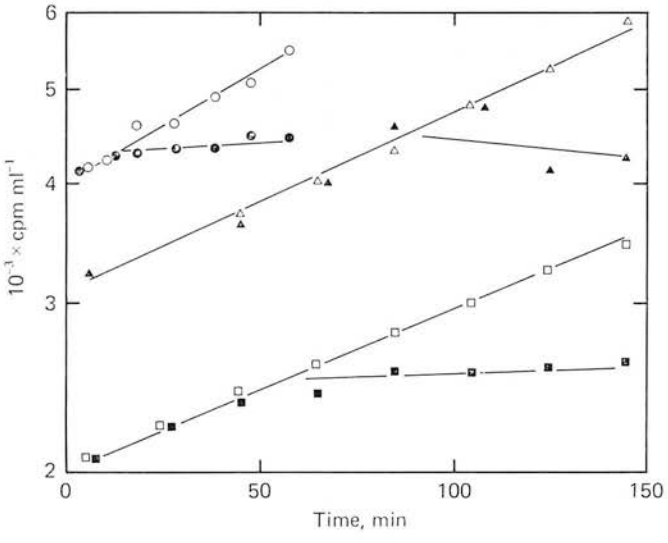

Fig. 5. Changes in rates of total RNA synthesis in asynchronous exponential phase cultures of 3 diploid strains of *S. pombe*, after inhibition of DNA synthesis. Initial density of cultures was between 0·2 and 1 × 10^6 cells/ml. ○, ●, wild type/wild type diploid cells; □, ■, *wee* 1–50/wild type diploid cells; △, ▲, *wee* 1–50/*wee* 1–50 diploid cells. Cultures were split at time 0; hydroxyurea was added to one half of each culture to a final concentration of 11 mM. Closed symbols show rates of RNA synthesis in hydroxyurea-treated subcultures. Open symbols show rates of RNA synthesis in control, untreated cultures.

In heterozygous *wee* 1–50/wild type cells, there was a delay of 50 to 70 min, or about 0·45 of a cell cycle, between addition of hydroxyurea and the onset of a constant rate of RNA synthesis.

These results are consistent with the following model: the stepwise doublings in rates of rRNA and poly(A)$^+$mRNA synthesis in each cell cycle are dependent on the previous round of DNA replication. However, in the case of the homozygous *wee* 1–50 and heterozygous *wee* 1–50/wild type diploids, there is a fraction of the cell cycle during which the cell has completed replication of its DNA, but has yet to double its rate of transcription. In asynchronous cultures (Fig. 5), there will be a proportion of cells in this state: these account for the period after addition of hydroxyurea, during which the rate of RNA synthesis rose as in the controls. It is consistent with this

argument that for each of the 3 strains, the length of the delay between addition of hydroxyurea and the onset of a constant rate of RNA synthesis was similar to the period between the mid-points of DNA replication and the following step-doublings in rates of RNA synthesis in synchronous cultures.

Hydroxyurea is the only inhibitor of yeast DNA synthesis known to produce the very rapid inhibition of DNA synthesis required for these experiments. It does, however, suffer from the drawback that it has been shown to inhibit cell growth and precursor uptake at concentrations only a little higher than required for inhibition of DNA synthesis (Mitchison & Creanor, 1971). In our experiments, the rate of adenine uptake in hydroxyurea-treated cultures did not deviate from that in control cultures until well after the onset of a constant rate of RNA synthesis. There was also no effect on general growth of the culture, as measured by optical density, until long after the effects on RNA synthesis had been noted. However, because of possible side effects, we cannot entirely exclude the possibility that the constant rates of RNA synthesis came about indirectly, through inhibition of some other aspect of metabolism by hydroxyurea.

DISCUSSION

The 3 diploid strains had different mean sizes and consequently different mean DNA concentrations (Tables 1, 2). DNA replication in the *wee* 1–50 diploid occurred later in the cell cycle than in the wild type or heterozygous diploid strains (Figs. 1–3). The timings of DNA replication in the 3 strains are consistent with the hypothesis that DNA replication cannot take place in cells which have not completed mitosis and which are beneath a minimum size (Nurse & Thuriaux, 1977). Further evidence for this hypothesis will be discussed elsewhere (K. Nasmyth, P. Nurse & R. S. S. Fraser, in preparation). The delayed DNA replication in *wee* 1–50 diploid cells served partly but not completely to compensate for the increased DNA concentration caused by the small mean size of cells of this strain.

Despite the 1·4-fold variation in gene concentration in the 3 diploid strains, the concentrations of total RNA and poly(A)$^+$mRNA were the same in all 3 lines. In all 3 strains, the rates of rRNA and poly(A)$^+$mRNA synthesis increased as a step once in each cell cycle, but the timing of the steps differed in the different strains. The smaller the cell at division, the later in the cell cycle were the steps in rates of rRNA and poly(A)$^+$mRNA synthesis.

The following paper (Barnes *et al.* 1978) shows that the observed differences in time of these rate doublings in the 3 strains are sufficient to maintain the same mean total RNA and poly(A)$^+$mRNA concentrations in cells of these different sizes. In this discussion we are concerned with 2 questions: what determines the cell cycle stage at which the doubling in rate of synthesis occurs, and what mechanism fixes the synthetic rate before and after the rate doubling.

Cell cycle control of transcription

Cell size-monitoring mechanisms have been proposed as controls of initiation of DNA synthesis and mitosis (Fantes *et al.* 1975). The observed correlation between cell

size and cell cycle stage of doubling in rate of RNA synthesis suggested that some aspect of cell size might also be involved in the control of initiation of the rate increase. We have examined this quantitatively by taking total protein as an index of cell size. Knowing the mean protein content per cell in exponential, asynchronous culture, and knowing that total protein increases close to exponentially during the cell cycle (Stebbing, 1971), we have calculated the protein per cell at the time of the mid-point of each doubling in rate of poly(A)$^+$mRNA synthesis (Table 3). For the 3 diploid strains, the doublings in rate of poly(A)$^+$mRNA synthesis occurred when the cells had very similar protein contents.

Table 3. *Mean protein per cell values at various stages of the cell cycles of* 3 *diploid and* 2 *haploid strains of S. pombe, and cell cycle stage of mid-point in doubling in rate of poly(A)$^+$mRNA synthesis in each strain*

Strain	pg protein/cell at		Cell cycle stage of mid-point in doubling of rate of poly(A)$^+$mRNA synthesis	Protein content at mid-point of doubling of rate of poly(A)$^+$mRNA synthesis	
	Start of cycle	End of cycle		pg/cell	pg/haploid genome
Diploids					
Wild type/wild type	16·3	32·6	0·03 ± 0·02	16·6 ± 0·2	8·3 ± 0·1
wee 1–50/wild type	13·3	26·6	0·50 ± 0·03	18·9 ± 0·3	9·5 ± 0·2
wee 1–50/*wee* 1–50	9·0	18·8	1·04 ± 0·04	19·0 ± 0·6	9·5 ± 0·3
Haploids					
Wild type	8·8	17·5	0·11 ± 0·04	9·5 ± 0·2	9·5 ± 0·2
wee 1–50	5·0	10·0	0·81 ± 0·06	8·7 ± 0·4	8·7 ± 0·4

Protein values were calculated from the measured mean protein content of cells in asynchronous exponential growth, using the cell age-distribution equation, and knowing that protein rises close to exponentially during the cell cycle. Time of mid-point of doubling in rate of poly(A)$^+$mRNA synthesis was measured in synchronous cultures (Figs. 1–3) and expressed in cell cycle units. Protein content per haploid genome for the diploid strains was obtained by dividing the value per cell by 2. Values are means ± standard errors. Values for haploid strains are from Fraser & Nurse, 1978.

The wild type and *wee* 1–50 haploid lines, for comparison, doubled their rates of poly(A)$^+$mRNA synthesis when their protein contents were quite different from the three diploid lines (Table 3; haploid data from Fraser & Nurse, 1978). However, if the protein contents per cell at the time of rate doubling are expressed per haploid genome, i.e. by dividing the diploid values by 2, it is clear that all 5 strains, diploid or haploid, doubled their rate of poly(A)$^+$mRNA synthesis when at a very similar protein content (Table 3). Similarly, the doubling in rate of rRNA synthesis also occurred in cells of a similar protein content per haploid genome, although the data available are less extensive. These results suggest that the rate doublings are triggered when the cells reach a threshold size, and that the controlling aspect of size is monitored on a per haploid genome basis. The comparison of haploid and diploid lines suggests that monitoring mechanisms based on surface area to volume ratios can be excluded.

One type of mechanism which would fit our results is the inhibitor-dilution model, such as has been proposed for the control of initiation of DNA replication in *Escherichia coli* by Pritchard, Barth & Collins (1969). A pulse of inhibitor is synthesized at a particular point in the cell cycle, for example at DNA replication or nuclear division. When diluted by cell growth to below a threshold level, the doubling in rate of transcription is initiated. If the amount of inhibitor synthesis per cell is constant, and the inhibitor is stable, then all cells will have to grow to the same size to reduce the inhibitor concentration to the same threshold level. Comparison of diploid and haploid lines suggests that the amount of inhibitor synthesized must be a constant amount per haploid genome. This would occur if inhibitor synthesis were gene-dosage dependent, and if the inhibitor genes were only available for transcription for a limited period during each cell cycle.

An alternative mechanism is that some controlling component is synthesized at a rate proportional to cell mass (Sompayrac & Maaløe, 1973). When a certain amount of this component has been accumulated per cell the events leading to the doubling in rates of RNA synthesis are set in motion. If the amount that has to be accumulated is double in a diploid then the doubling in rate of RNA synthesis will take place in cells of a constant size per haploid genome.

We do not know the biochemical mechanism by which a cell mass-related control could alter the rates of RNA synthesis. It is even possible that the mass control acts on some separate component of cell metabolism, and that the induced cyclic behaviour of that component entrains the periodic changes in rates of RNA synthesis (Goodwin, 1963).

Mechanisms setting the rates of RNA synthesis

The cell cycle patterns of RNA synthesis in the 3 diploid strains may be interpreted in terms of a model outlined briefly elsewhere (Fraser & Nurse, 1978). This model proposes that the rates of synthesis of rRNA and the majority of polyadenylated mRNAs are under a general control, perhaps analogous to stringent control of RNA synthesis in bacteria (Cashel & Gallant, 1974). This control sets the rates at a certain level at the beginning of the cycle, and at twice that rate when the cell attains a threshold size.

The dependency of the doubling in rates on the previous round of DNA replication suggests that the setting of the rate at each level may be based ultimately on gene dosage. However, the rate cannot depend solely on gene dosage, as in *wee* 1–50 diploid and heterozygous diploid cells, a considerable delay occurs between DNA replication and doubling in the rate of RNA synthesis. There must be some mechanism in these strains to keep the rate of RNA synthesis at the lower level during the period between replication of the genome and the attainment of a critical cell size.

One possibility would be that half of the copies of each gene present after DNA replication might be completely inactivated until the cell reached the threshold size. Such inactivation could be caused by DNA modification (Holliday & Pugh, 1975), by chromosomal folding (Vaughan, 1977) or by chromosomal proteins (Paul & Gilmour, 1968). An analogous form of control exists over those genes situated on the X chromosome in mammals. In females, with 2 X chromosomes and hence twice as many X

chromosome genes as males, compensation for the doubled concentration of these genes is by complete inactivation of one of the 2 X chromosomes (Lyon, 1961, 1972). In this context, it is interesting that in the early female mammalian embryo both X chromosomes are active (Monk & Kathuria, 1977). Only later, during cell cleavage when the cells get smaller is one of the X chromosomes inactivated. Perhaps when the cells are reduced beneath a certain critical size, a mechanism similar to that observed in *S. pombe* operates to inactivate one of the X chromosomes.

Alternatively, the rate of transcription might be maintained at the pre-DNA replication rate if the 2 copies of each gene present after replication were transcribed, on average, at half the rate of transcription of the single copy present before replication. On reaching the threshold size, the rate of transcription of both copies would double, to equal that of the single copy present before replication. A similar mechanism exists in dosage compensation of the X chromosome in *Drosophila* (Lucchesi, 1973; Courtright, 1976). In this organism, the 2 genes present on the 2 X chromosomes in the female are each expressed at half the rate of the single gene present on the single X chromosome in the male. The means by which the rate of transcription per gene could be restricted in *S. pombe* to half of the rate before DNA replication are not clear. The mechanism could involve limited availability of the gene, or control by altered activity of RNA polymerase or some other component of transcription.

We have discussed 3 examples from eukaryotes of mechanisms compensating for increased gene concentrations. All operate by effectively reducing the mean concentration of active DNA. In the prokaryote *Escherichia coli*, a mechanism compensating for a reduced gene concentration has been proposed. Chandler & Pritchard (1975) suggested that when gene concentration is reduced, the rate of total protein synthesis is maintained by lowering the level of repression of those genes subject to specific controls at the transcriptional level.

Elsewhere, we have considered how a cell size-controlled doubling in rate of RNA synthesis may be of widespread importance in the control of enzyme accumulation and balanced exponential growth of *S. pombe* cells (Fraser & Moreno, 1976; Fraser & Nurse, 1978; Barnes *et al.* 1978). It should be emphasized, however, that the proposed general size control over rate of messenger RNA synthesis is obviously not the only mechanism controlling cell growth and enzyme synthesis in *S. pombe*. Some genes, such as those associated with periodic events like DNA replication, may be transcribed periodically. Transcription of some is induced by alteration in nutrient conditions (Mitchison & Creanor, 1969). There is also evidence that synthesis of some enzymes may be regulated at the translational level (Creanor, May & Mitchison, 1975; Fraser, 1975). The proposed cell size-related general control over gene activity also does not exclude the possibility that genes are individually subject to further, specific controls.

Limits of the compensation mechanism in S. pombe

If the doubling in rate of RNA synthesis is dependent on DNA replication, there are limits to the cell size range over which the compensation mechanism will function effectively. In cells of wild type size, DNA replication and thus the doubling in rate of RNA synthesis cannot occur significantly earlier in the cycle, as DNA replication

cannot take place before completion of mitosis (Nurse & Thuriaux, 1977). Any increase in mean cell size at division of such cells would reduce the gene concentration and the steady-state RNA concentration.

The mechanism of the size control initiating the doubling in rate of RNA synthesis may also set a limit to how small a cell can be. For example, if the rate doubling is triggered when a certain amount of component has been accumulated per cell, since division will halve that amount, a cell which has not reached the threshold level of component at division will never do so. With this mechanism the smallest cell which could maintain a constant mean concentration of RNA would be about half the size of wild type. A similar minimum cell size would also apply to the inhibitor-dilution model if the inhibitor were made at a constant time in the cell cycle, say just after cell separation. However, if the inhibitor were made at the time of DNA replication, which takes place later in the cycle in small cells, the minimum cell size would be less than half that of wild type.

The nature of the mechanism limiting the rate of transcription between DNA replication and doubling in rate of RNA synthesis may also have a bearing on minimum cell size. If one copy of each gene is inactivated until the threshold size is reached, and the cell undergoes nuclear division beneath this size, then by independent segregation of sister chromatids each daughter nucleus would have an incomplete complement of active genes. This is likely to be a non-viable situation, and would place a lower limit on viable cell size.

In this context, it is interesting to return to the behaviour of the *wee* 1–50 diploid in synchronous culture. The interval between successive divisions in synchronous culture of *wee* 1–50 diploid cells was the same as in wild type diploids, while in asynchronous culture the mean cell number doubling time of the *wee* 1–50 diploid was much longer than that of wild type diploids. The increase in cell number at each division in synchronous cultures of *wee* 1–50 diploids was less than a doubling. These results show that in *wee* 1–50 diploid synchronous cultures, a fraction of cells lost viability in each cell cycle. Cells at the end of their cell cycle will show a variation in size: it is possible that the smallest cells were too small to activate the doubling in rate of RNA synthesis before division, and thus became non-viable. These cells would also explain the failure of synchronous cultures of *wee* 1–50 diploids to double their rates of rRNA and poly(A)$^+$mRNA synthesis in each cycle. It may be relevant to this argument that mutants smaller than *wee* 1–50 have not yet been found.

This work was supported by the Medical Research Council and the Science Research Council. We thank Robert Pitcairn and Laurence Errington for technical assistance, and Murdoch Mitchison for useful discussions.

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(*Received* 8 *May* 1978)

J. Cell Sci. 35, 41–51 (1979)
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ANALYSIS OF THE SIGNIFICANCE OF A PERIODIC, CELL SIZE-CONTROLLED DOUBLING IN RATES OF MACROMOLECULAR SYNTHESIS FOR THE CONTROL OF BALANCED EXPONENTIAL GROWTH OF FISSION YEAST CELLS

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SUMMARY

Mutant strains of the fission yeast *Schizosaccharomyces pombe* are available which divide at smaller mean sizes than wild type. Earlier work by the present authors has shown that all these strains double their rates of polyadenylated messenger RNA synthesis as a step once in each cell cycle. The smaller the cell, the later in the cycle is the doubling in rate of synthesis. Strains of all sizes, however, double their synthetic rate when at the same threshold size. We show here that the differences in cell cycle stage of doubling in rate of polyadenylated messenger RNA synthesis are enough to explain the reduced mean steady state polyadenylated messenger RNA contents of the smaller strains. The cell size-related control over doubling in rate of synthesis is also shown to maintain the mean polyadenylated messenger RNA content as a constant proportion of cell mass, irrespective of cell size. This control thus allows cells to maintain balanced exponential growth, even when absolute growth rate per cell is altered by mutation. It is also shown that the concentration of polyadenylated messenger RNA itself could act as a monitor of the threshold size triggering the doubling in rate of synthesis in each cell cycle.

INTRODUCTION

During balanced exponential growth of cell cultures, individual parameters of growth remain at a constant proportion of total cell mass. At the level of the individual cell, the amount of each cell component must on average double during each cell cycle, so that the daughter cells formed at division are identical to the parent at the same stage in the preceding cycle. One mechanism which can account for the doubling in amount of a component is a stepwise doubling in the rate of synthesis of that component at a fixed point in each cell cycle. In the fission yeast *Schizosaccharomyces pombe*, there are periodic doublings once in each cell cycle in the rates of synthesis of several enzymes (Mitchison & Creanor, 1969), ribosomal RNA (rRNA) (Wain & Staatz, 1973; Fraser & Moreno, 1976) and polyadenylated messenger RNA (poly (A)$^+$mRNA) (Fraser & Moreno, 1976; Fraser & Nurse, 1978*a*).

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One approach to the problem of how individual growth parameters may be maintained at a constant mean proportion of cell mass has been made possible by the discovery of mutants of fission yeast which divide at different mean sizes from wild type (Nurse, 1975). Fraser & Nurse (1978*a*, *b*) showed that the smaller the cell, the later in the cell cycle was the stepwise doubling in rate of poly(A)$^+$mRNA or rRNA synthesis. In this paper we will show that the observed delay in the doubling in rate of poly(A)$^+$mRNA synthesis in the cell cycle is enough to account for the reduced poly(A)$^+$mRNA content of the smaller cells (Fraser & Nurse, 1978*a*). Furthermore, if the doublings in synthetic rate always occur in cells of the same size, then poly(A)$^+$mRNA content will be maintained at a constant proportion of cell mass irrespective of cell size or absolute growth rate per cell. We also show that the concentration of poly(A)$^+$mRNA itself could act as a signal for the onset of doubling in rate of poly(A)$^+$mRNA synthesis in each cell cycle.

Table 1. *Volumes at division and mean protein contents of 5 strains of Schizosaccharomyces pombe*

Strain	Mean cell vol. at division, μm^3	Mean protein per cell, pg ± S.E.M.
Haploids		
Wild type	149	12·1 ± 0·3
wee 1–50	73	6·9 ± 0·4
Diploids		
Wild type/wild type	283	22·6 ± 0·5
wee 1–50/wild type	231	18·5 ± 0·8
wee 1–50/*wee* 1–50	144	13·0 ± 0·2

MATERIALS AND METHODS

Wild type cells were strain 972 h$^-$ of *Schizosaccharomyces pombe* Lindner. The derived mutant *wee* 1–50 (Nurse, 1975) has an alteration in the control over initiation of nuclear division, such that it undergoes nuclear division and cell division at slightly more than half the size of wild type. Cell cycle data were obtained for haploid wild type and *wee* 1–50 (Fraser & Nurse, 1978*a*) and for 3 diploid strains: wild type and *wee* 1–50 homozygous diploids and the heterozygous *wee* 1–50/wild type diploid (Fraser & Nurse, 1978*b*). The 3 diploid and 2 haploid strains together cover a size range from approximately 0·6 to 1·9, relative to the haploid wild type size of 1·0 (Table 1).

RESULTS AND DISCUSSION

Table 2 summarizes the relative poly(A)$^+$mRNA contents of the 3 diploid and 2 haploid strains studied, and the times in their cell cycles when the rate of poly(A)$^+$mRNA synthesis underwent a stepwise doubling. It is clear that in both haploid and diploid series, the smaller the cell, the lower the relative content of poly(A)$^+$mRNA per cell, and the later in the cell cycle the doubling in rate of poly(A)$^+$mRNA synthesis.

We wish first to establish that the delay between doubling in rate of poly(A)$^+$mRNA synthesis in wild type and smaller mutants is all that is required to account for the

reduced mean poly(A)$^+$mRNA content of the smaller cells. This will be done by calculating how much the poly(A)$^+$mRNA content of the smaller cells would be, if the delay in doubling of the synthetic rate were the only difference between the 2 strains, and comparing this with the experimentally measured ratio.

Table 2. *Mid-points of doublings in rate of poly(A)$^+$mRNA synthesis, and poly(A)$^+$mRNA contents of 5 strains of Schizosaccharomyces pombe*

Strain	Cell cycle stage of mid-point in doubling of rate of poly(A)$^+$mRNA synthesis	l	Poly(A)$^+$mRNA content per cell as a percentage of wild type of the same ploidy level	
			Calculated	Measured
Haploids				
Wild type	0·11 ± 0·04	—	—	—
wee 1–50	0·81 ± 0·06	0·70 ± 0·07	62 ± 2	59 ± 3
Diploids				
Wild type/wild type	0·03 ± 0·02	—	—	—
wee 1–50/wild type	0·50 ± 0·03	0·47 ± 0·04	72 ± 2	80 ± 4
wee 1–50/*wee* 1–50	1·04 ± 0·04	1·01 ± 0·05	50 ± 2	57 ± 2

Mid-points of doublings in rate of poly(A)$^+$mRNA synthesis were measured in synchronous cultures (data from Fraser & Nurse, 1978*a*, *b*). l is the time, in cell cycle units, between the mid-point of doubling in a small mutant cell and in wild type of the same ploidy. Poly(A)$^+$mRNA content of small mutant cells was calculated as 2^{-l} of wild type content, as explained in the text. All values are means ± standard errors.

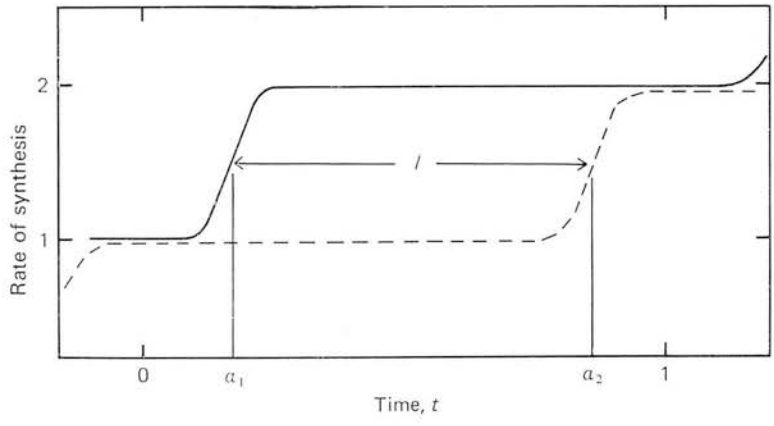

Fig. 1. The assumed patterns of rates of synthesis of poly(A)$^+$mRNA in wild type (——) and small mutant *wee* 1–50 (– – –) cell cycles. Wild type cells are assumed to synthesize poly(A)$^+$mRNA at a rate f(t) (——), and *wee* 1–50 cells at a rate f($t-l$) (– – –). α_1 and α_2 are the cell cycle stages of the mid-points of the doublings in rate of poly(A)$^+$mRNA synthesis in wild type and *wee* 1–50 cells respectively. l is the time between the mid-points of doubling in rates of poly(A)$^+$mRNA synthesis in wild type and *wee* 1–50, expressed as a fraction of the cell cycle. One time equals a complete cell cycle.

Derivation of an expression for the average poly(A)⁺mRNA content of a mutant small cell compared to wild type

Consider first the rates of poly(A)⁺mRNA synthesis in synchronously dividing populations of wild type and small mutant *wee* 1–50 cells. The cell cycle commences with the same absolute rate of poly(A)⁺mRNA synthesis per cell in each strain (Fraser & Nurse, 1978*a*). The rate of synthesis in wild type cells increases as a function of time $f(t)$, so that at the end of the cell cycle it is exactly double the initial rate (Fig. 1). In *wee* 1–50 cells, the rate of synthesis of poly(A)+mRNA follows the same pattern as in wild type, except for a delay of a fraction l of a cell cycle before the rate of poly(A)+mRNA synthesis doubles. Cell cycle patterns of poly(A)+mRNA synthesis such as shown in Fig. 1 have been demonstrated experimentally for wild type and *wee* 1–50 cells (Fraser & Nurse, 1978*a*). However, the following proof is valid for any pattern of increase during the cell cycle which is the same for wild type and *wee* 1–50.

Let R' and R'_m be the amounts of poly(A)⁺mRNA in synchronously dividing populations of wild type and *wee* 1–50 cells; R' and R'_m depend on time t, which is measured in cell cycle units (one cycle or mean generation time = 1·0 cell cycle unit). The degradation of poly(A)⁺mRNA has been shown to follow approximately first-order kinetics (Fraser, 1975); we assume that the half-life is the same in both strains. Thus the net rates of poly(A)⁺mRNA accumulation are given by:

$$\frac{dR'}{dt} = N(t)f(t) - \lambda R' \quad \text{for wild type}$$

$$\frac{dR'_m}{dt} = N(t)f(t-l) - \lambda R'_m \quad \text{for } wee \text{ 1–50,} \tag{1}$$

where λ is the degradation rate constant, $N(t)$ is the number of cells in the population at any time t, and $f(t)$ is the rate of synthesis of poly(A)⁺mRNA in wild type cells; $f(t-l)$ is the rate in *wee* 1–50 cells (Fig. 1).

In addition, as the populations double in each cell cycle unit of time, but at any time consist of cells identical to those of the previous generation at the same stage in the cell cycle, we may write

$$f(t) = 2f(t-1). \tag{2}$$

Now consider asynchronous, exponentially growing populations of wild type and *wee* 1–50 cells, with the same numbers of cells per ml, and growing with the same mean generation time (Nurse, 1975). R and R_m are the amounts of poly(A)⁺mRNA in the wild type and mutant populations respectively. From the canonical cell age distribution equation (Cook & James, 1964) the fraction of cells aged α to $\alpha+\delta\alpha$ is given by

$$\ln 2 . 2^{1-\alpha}\delta\alpha \tag{3}$$

at all times. From Fig. 1 it is clear that the rates of synthesis of poly(A)⁺mRNA for cells aged α are given by $f(\alpha)$ and $f(\alpha-l)$ for wild type and *wee* 1–50 respectively. By

integrating these rates of synthesis, weighted for cell age, over all possible ages, we obtain the average rate of synthesis of poly(A)$^+$mRNA in asynchronous population as

$$\left.\begin{aligned} &\int_0^1 \mathrm{f}(\alpha).\ln 2.2^{1-\alpha}\,\mathrm{d}\alpha \quad \text{in wild type} \\ \text{and}\quad &\int_0^1 \mathrm{f}(\alpha-l).\ln 2.2^{1-\alpha}\,\mathrm{d}\alpha \quad \text{in } \textit{wee } 1\text{–}50. \end{aligned}\right\} \tag{4}$$

As the number of cells in each population is increasing exponentially, the total number of cells may be written as $N = N_0\,\mathrm{e}^{kt}$ for both populations, where N_0 and k are constants. The total rates of synthesis of poly(A)$^+$mRNA in each population are given by the total number of cells multiplied by the average rates of synthesis.

We may now write analogous equations to (1) for rates of accumulation of poly(A)$^+$mRNA in asynchronous, exponentially-growing populations:
for wild type

$$\frac{\mathrm{d}R}{\mathrm{d}t} = C\int_0^1 \mathrm{f}(\alpha)2^{1-\alpha}\,\mathrm{d}\alpha - \lambda R \tag{5a}$$

and for *wee* 1–50

$$\frac{\mathrm{d}R_m}{\mathrm{d}t} = C\int_0^1 \mathrm{f}(\alpha-l)2^{1-\alpha}\,\mathrm{d}\alpha - \lambda R_m, \tag{5b}$$

where $C = N_0\,\mathrm{e}^{kt}.\ln 2$.

Equation (5*b*) may be written as

$$\frac{\mathrm{d}R_m}{\mathrm{d}t} = C.2^{-l}\int_0^1 \mathrm{f}(\alpha-l)2^{1-\alpha+l}\,\mathrm{d}(\alpha-l) - \lambda R_m \tag{6}$$

and since f is a periodic function integrated over a whole period, (6) may be rewritten as

$$\frac{\mathrm{d}R_m}{\mathrm{d}t} = C.2^{-l}\int_0^1 \mathrm{f}(\alpha)2^{1-\alpha}\,\mathrm{d}\alpha - \lambda R_m$$

Therefore, writing

$$B = \int_0^1 \mathrm{f}(\alpha)2^{1-\alpha}\,\mathrm{d}\alpha$$

equations (5) may be rewritten as

$$\left.\begin{aligned} &\frac{\mathrm{d}R}{\mathrm{d}t} = C.B - \lambda R \\ \text{and}\quad &\frac{\mathrm{d}R_m}{\mathrm{d}t} = C.B.2^{-l} - \lambda R_m. \end{aligned}\right\} \tag{7}$$

As the numbers of cells in the 2 populations are increasing at the same exponential rate and as the age distribution of the population remains fixed, the amount of poly(A)$^+$mRNA in the 2 populations must also increase at the same exponential rate. So

$$\left.\begin{aligned} &\frac{\mathrm{d}R}{\mathrm{d}t} = qR \\ &\frac{\mathrm{d}R_m}{\mathrm{d}t} = qR_m, \end{aligned}\right\} \tag{8}$$

where q is a constant.

Combining equations (7) and (8) by eliminating $\frac{dR}{dt}$ and $\frac{dR_m}{dt}$ and rearranging gives

$$\left.\begin{aligned}(q+\lambda)R &= C.B \\ \text{and} \qquad (q+\lambda)R_m &= C.B.2^{-l}\end{aligned}\right\} \tag{9}$$

from which it follows directly that

$$\frac{R_m}{R} = 2^{-l}. \tag{10}$$

This result may be more readily appreciated from the following simpler explanation, which is not, however, a formal mathematical proof. Consider populations of wild

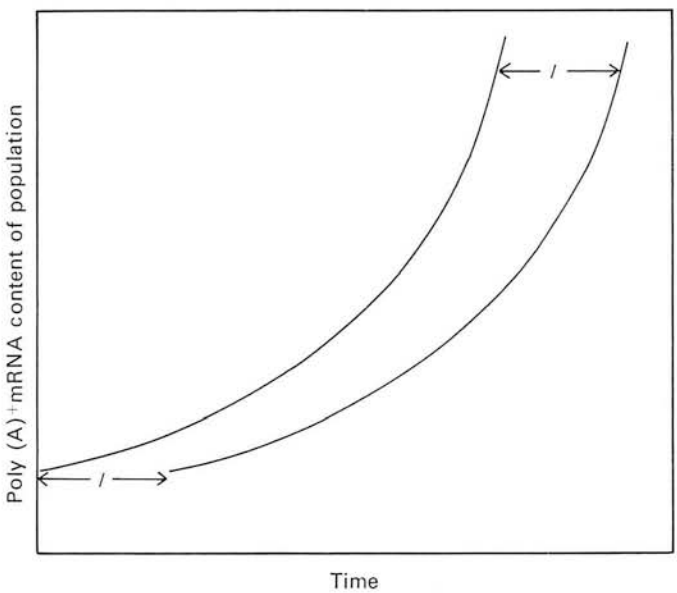

Fig. 2. Poly(A)$^+$mRNA contents of asynchronous, exponentially growing populations of wild type (upper curve) and *wee* 1–50 cells (lower curve). The 2 populations contain the same number of cells at any time. The fixed time shift, of magnitude l between the curves, is a consequence of the delay, of the same magnitude, between the doubling in rate of poly(A)$^+$mRNA synthesis in the wild type and *wee* 1–50 cell cycles.

type and *wee* 1–50 containing the same numbers of cells and growing at the same exponential rate. The delay, of a fraction l of the cell cycle, in the doubling of the rate of synthesis of poly(A)$^+$mRNA in *wee* 1–50 cells (Fig. 1) implies that the amount of poly(A)$^+$mRNA in the *wee* 1–50 population will lag behind that of the wild type population by a time interval l, as shown in Fig. 2. Thus if the *wee* 1–50 population contained a particular amount of poly(A)$^+$mRNA at a particular time t, the wild type population would have contained the same amount at time $t-l$. In the intervening interval both populations would have increased in cell number by a factor 2^l. Thus the average amount of poly(A)$^+$mRNA per cell in the *wee* 1–50 population at any time will be 2^{-l} of that in the average cell of the wild type population.

Using this expression, and the values for l measured in synchronous cultures for the haploid and diploid series of *S. pombe*, we have calculated mean relative poly(A)$^+$mRNA contents for the small mutant cells of each series as a fraction of wild type. Table 2 (p. 43) shows that these calculated values are in good agreement with the values determined experimentally in asynchronous, exponentially growing cultures.

We conclude that delaying the doubling in rate of poly(A)$^+$mRNA synthesis in the cell cycle is alone sufficient to account for the reduced poly(A)$^+$mRNA content of those cells forced by mutation to be of smaller mean size than wild type.

Delayed doubling in the rate of poly(A)$^+$mRNA synthesis in small mutant cells keeps the ratio of the average poly(A)$^+$mRNA content to average total cell mass the same as in wild type

It has been shown experimentally for the haploid and diploid series that at the time of the mid-point of doubling in rate of poly(A)$^+$mRNA synthesis, the members of each series have similar protein contents per cell (Fraser & Nurse, 1978*b*). We have taken protein content per cell as a measure of cell size, as it is easy to measure accurately. Protein increases close to exponentially through the cell cycle (Stebbing, 1971) and it is likely that other parameters of growth such as total cell mass or volume will also increase close to exponentially through the cell cycle. We shall refer to any of these parameters representing overall cell size as V, and will demonstrate that if the value of V is the same for wild type and small mutant cells at the time of the mid-point in doubling of the rate of poly(A)$^+$mRNA synthesis, then the ratio of the average poly(A)$^+$mRNA content to average V in asynchronous, exponentially growing populations must be the same for strains of all sizes.

Let $V(\alpha)$ and $V_m(\alpha)$ be the value of the growth parameter (be it protein, mass or volume) of cells aged α, of wild type and small mutants respectively. Both $V(\alpha)$ and $V_m(\alpha)$ increase exponentially with the same specific rate of increase. This rate being such that the cell sizes at $\alpha = 1$ must be twice the sizes at $\alpha = 0$, we may write:

$$V(\alpha) = V(0)2^{\alpha} \tag{11a}$$

and

$$V_m(\alpha) = V_m(0)2^{\alpha}. \tag{11b}$$

Let the cell age at the mid-point of the doubling in rate of poly(A)$^+$mRNA synthesis be α_1 in wild type cells and α_2 in small mutant cells; so that $\alpha_2 = \alpha_1 - l$ (Fig. 1). The property of cell size parameters assumed for the proof implies that

$$V(\alpha_1) = V_m(\alpha_2). \tag{12}$$

Substituting α_1 in (11*a*) and α_2 in (11*b*) and making use of (12) it follows that

$$V(0) = V_m(0)2^l \tag{13}$$

Using (13) to eliminate $V(0)$ and $V_m(0)$ from (11) leads to

$$V(\alpha) = V_m(\alpha)2^l. \tag{14}$$

In asynchronous, exponentially growing populations, cell age distribution remains

fixed and is the same for wild type and small mutant populations. As equation (14) implies that the wild type cells are always 2^I larger than the mutant of corresponding age, it follows directly that the average cell size in a wild type population will always be 2^I larger than in small mutant populations. From equation (10) the average amount of poly(A)$^+$mRNA in wild type cells is also 2^I greater than in mutant cells at all times. Therefore, it follows directly that the ratio of average poly(A)$^+$mRNA content to average cell size must be the same for wild type and small mutant strains.

From this proof, we can conclude that the control of the cell cycle stage of doubling in the rate of poly(A)$^+$mRNA synthesis by a threshold cell size which is the same for mutants of a range of mean sizes (Fraser & Nurse, 1978*a*, *b*) will maintain average poly(A)$^+$mRNA content at a constant proportion of total cell protein, mass or volume during growth, irrespective of cell size or absolute growth rate per cell. This control therefore acts homeostatically: when the growth of the cell is distorted by the presence of the *wee* 1 mutation, leading to an altered absolute growth rate per cell, the size-related control is nonetheless able to maintain balanced growth and keep poly(A)$^+$mRNA content in line with total cell growth. A further property of the cell-size control over doubling in rate of poly(A)$^+$mRNA synthesis is that it enables cells to compensate for variation in gene concentration. For example, the 3 diploid strains have different gene concentrations but the size control over doubling in rate of RNA synthesis allows them to grow with the same mean RNA concentration (Fraser & Nurse, 1978*b*).

The concentration of poly(A)$^+$mRNA could itself act as the trigger initiating the doubling in rate of poly(A)$^+$mRNA synthesis in each cell cycle

The 3 diploid strains of different mean sizes all double their rate of poly(A)$^+$mRNA synthesis at points in their cell cycles when they have very similar protein contents per cell (Fraser & Nurse, 1978*b*). Similarly, the 2 haploid strains have similar protein contents per cell when they double their rates of poly(A)$^+$mRNA synthesis (Fraser & Nurse, 1978*a*). All 5 strains double their rate of poly(A)$^+$mRNA synthesis when they have similar protein contents per haploid genome per cell, suggesting that the timing of the doubling in rate of synthesis involves monitoring of some aspect of cell size. We wish now to establish that the concentration of poly(A)$^+$mRNA itself could act as a size-monitoring mechanism.

As poly(A)$^+$mRNA is unstable, if it is synthesized at a constant rate per cell the content per cell will approach a steady state. Doubling the rate of synthesis as a discrete step once per cell cycle will lead to an increase in the poly(A)$^+$mRNA content, which will again tend to a steady state at a higher level (Fig. 3). We assume that cell volume increases exponentially, and that the half-life of poly(A)$^+$mRNA is 0·275 of a generation time (Fraser, 1975) in all strains. As the cells must exactly double their poly(A)$^+$mRNA content in one cell cycle, knowledge of the half-life is sufficient to determine the relative initial rates of synthesis of poly(A)$^+$mRNA. From these figures and the times of doubling in rates of synthesis in the cell cycle, the cell content of poly(A)$^+$mRNA can be calculated for any stage in the cell cycle. Dividing the

poly(A)$^+$mRNA content by the cell volume at that stage gives the poly(A)$^+$mRNA concentration.

Using experimentally measured values for total protein per cell as a measure of cell mass or volume, Fig. 4 shows calculated changes in poly(A)$^+$mRNA concentration during the cell cycles of the 3 strains for which we have the most extensive cell cycle

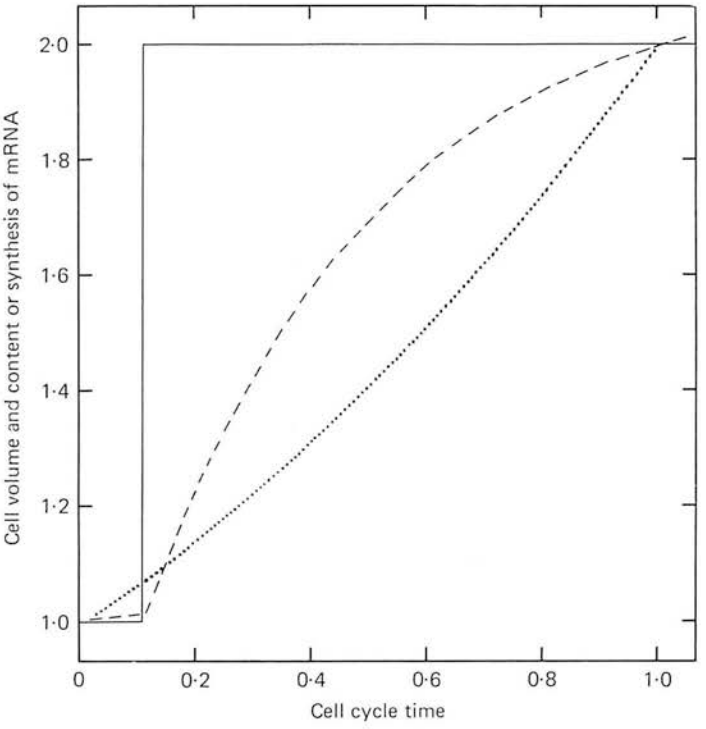

Fig. 3. Changes in rate of poly(A)$^+$mRNA synthesis (——) and in calculated poly(A)$^+$mRNA content per cell (– – –) during the cell cycle of ideal cells. The example shown is haploid wild type with a doubling in rate of poly(A)$^+$mRNA synthesis at 0·11 of the cell cycle (Fraser & Nurse, 1978*a*). The rate doubling is assumed to be instantaneous. ·····, change in cell volume (or mass, or protein) assumed to increase exponentially during the cell cycle.

data; all strains studied show the same basic pattern. In each curve, there is initially a decline in concentration, then at the time in the cell cycle when the rate of poly(A)$^+$mRNA synthesis doubles, the concentration of poly(A)$^+$mRNA begins to rise. The minimum concentrations of poly(A)$^+$mRNA reached in the different strains are very similar.

In addition to this experimentally based evidence, it can be shown theoretically that if exponentially growing cells from any 2 strains have the same volume at the time of doubling in rate of poly(A)$^+$mRNA synthesis, and if the rate of poly(A)$^+$mRNA synthesis follows the generalized pattern indicated in Fig. 1, then the minimum concentrations of poly(A)$^+$mRNA must be the same and must occur at the time when the rate of poly(A)$^+$mRNA synthesis doubles. This result may be proved by integrating equations (1) and making use of the property in equation (2).

This experimental and theoretical evidence therefore indicates that the concentration of poly(A)$^+$mRNA itself would be capable of acting in the mechanism which triggers the doubling in rate of poly(A)$^+$mRNA synthesis in each cycle. As the concentration of poly(A)$^+$mRNA in the cell depends not only on the rate of synthesis of poly(A)$^+$mRNA but also on cell growth, it follows that this mechanism is essentially a cell-size monitoring mechanism. A doubled rate of synthesis of poly(A)$^+$mRNA is switched on when the cell has grown to a sufficient size to reduce the concentration of poly(A)$^+$mRNA below a threshold level. Furthermore, as the trigger responds to a concentration and not to an absolute amount per cell, the mechanism would be able to operate in both haploid and diploid cells without further elaboration.

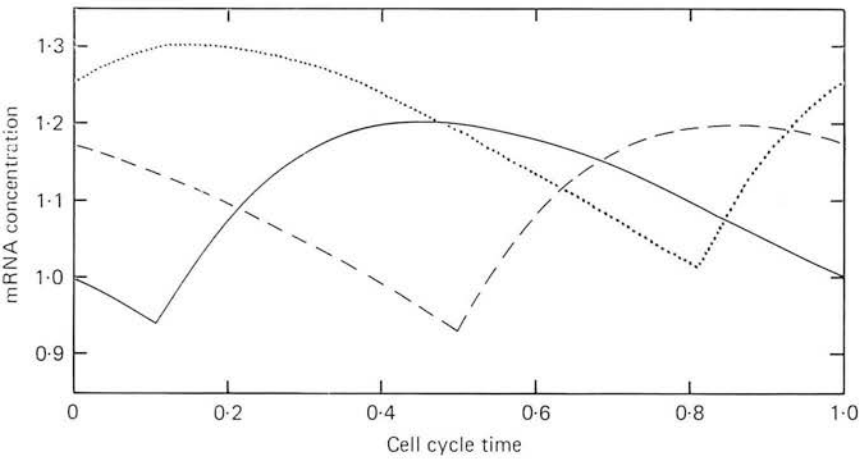

Fig. 4. Changes during the cell cycle in poly(A)$^+$mRNA concentration in 3 strains of *S. pombe*. Changes in cell content of poly(A)$^+$mRNA were calculated from the observed times of doubling of rate of poly(A)$^+$mRNA synthesis in the cell cycle (Fraser & Nurse, 1978*a*, *b*) as shown in Fig. 3. Poly(A)$^+$mRNA concentration was calculated by dividing poly(A)$^+$mRNA content by the cell size parameter (based on total protein estimations) at each stage of the cell cycle. ——, wild type haploid; ·····, *wee* 1–50 haploid; – – –, wild type/*wee* 1–50 heterozygous diploid.

CONCLUSION

In this paper we have analysed the behaviour of a component whose rate of synthesis doubles as a step during the cell cycle. We have shown that the component will be maintained on average at a constant proportion of mass in cells of different sizes at division, provided that the rate of synthesis per cell before the step doubling is the same in the different cells, and that the cell size at the time of the step doubling is the same in the different cells. We have used as an example the rate of synthesis of poly(A)$^+$mRNA, which doubles as a step. However, the proof will also apply to stable molecules such as rRNA, and to other components regardless of their actual patterns of increase through the cell cycle. As long as the rate of synthesis per cell and cell size are the same at any particular point on the pattern, the component will be maintained on average as a constant proportion of cell mass irrespective of actual cell mass.

Other cell components whose rates of synthesis are dependent upon a component

regulated by a control of the type we have described will also be maintained at a constant average proportion of cell mass. An example of this may be provided in *S. pombe* by 3 enzymes (Mitchison & Creanor, 1969) the accumulation of which may be dependent on mRNA content (Fraser & Moreno, 1976). Therefore a regulatory mechanism involving cell size control over rates of synthesis could be of widespread significance in the control of balanced exponential growth of cells.

This work was supported by the Agricultural, Science and Medical Research Councils. We thank Dr G. H. Freeman, Professor. J. K. A. Bleasdale and Professor J. M. Mitchison, F.R.S. for useful comments.

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(*Received* 8 *May* 1978)

J. Cell Sci. 39, 215–233 (1979)
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THE EFFECT OF CELL MASS ON THE CELL CYCLE TIMING AND DURATION OF *S*-PHASE IN FISSION YEAST

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SUMMARY

Two isotopic methods for measuring DNA replication in the fission yeast *Schizosaccharomyces pombe* are described. The first is a method for measuring the total quantity of [^{3}H]uracil incorporated into DNA after pulse labelling. The second is a means of detecting DNA replication in single cells by autoradiography. Both of these techniques have been used to investigate the timing and duration of *S*-phase in a series of mutant strains whose cell mass at division varies over a 3-fold range. The results support the hypothesis that in *S. pombe* there are 2 different controls over the timing of *S*-phase: an attainment of a critical cell mass and a dependency upon the completion of the previous mitosis coupled with a short minimum time in G_1. Strains whose cell mass at birth is above this critical level initiate DNA replication almost immediately after septation, that is, very soon after the previous mitosis. Strains whose cell mass at birth is below the critical level do not initiate replication until the critical cell mass is attained. The duration of *S*-phase has been estimated from the proportion of cells whose nuclei are labelled after a pulse of given duration. *S*-phase is short in *S. pombe*, lasting only about 0·1 of a cell cycle in wild type. Cell mass at *S*-phase does not have any consistent effect on this length. We have also investigated the degree of synchrony of *S*-phase initiation in daughter cells, and have found that, in a cell cycle 240 min long, their *S*-phases are initiated within 1–2 min of each other. This result indicates that between sisters variability in the duration of the G_1 phase is small compared with variability in the total cell cycle time, and argues against the hypothesis that the rate of cell cycle traverse is determined by a random transition in G_1.

INTRODUCTION

In eukaryotic cells DNA is replicated during a discrete period of the cell cycle known as the *S*-phase. It is not yet clear what factors are most important in determining the timing and duration of *S*-phase. In the fission yeast *Schizosaccharomyces pombe* we have proposed that the initiation of *S*-phase is subject to 2 controls: (1) the necessity for the cell to attain a particular minimal cell mass; and (2) the dependency of DNA replication upon completion of the previous mitosis (Nurse, 1975; Nurse, Thuriaux & Nasmyth, 1976; Nurse & Thuriaux, 1977); which of these 2 controls was operative in a particular cell was determined by the mass of that cell at its previous cell division. If cells divided at a small mass, then the requirement to attain a particular minimal cell mass determined the cell cycle timing of *S*-phase. If cells divided at a large mass,

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then it was the dependency of DNA replication upon the completion of mitosis coupled with a short minimum time in G_1 that determined the timing of *S*-phase. This hypothesis can be tested by investigating the timing of *S*-phase in mutant strains of *S. pombe* which undergo cell division at varying cell masses. Wild type and 4 mutant strains are available which vary in cell mass at division over a 3-fold range, with little change in generation time (Nurse, 1975; Thuriaux, Nurse & Carter, 1978; Nurse & Thuriaux, unpublished). In this paper we describe the use of these 5 strains to investigate the role cell mass plays in the cell cycle timing of *S*-phase. We also examine the effect cell mass has on the duration of *S*-phase, since some evidence suggests that the duration of *S*-phase is affected by cell mass (Callan, 1973; Blumenthal, Kriegstein & Hogness, 1973; Taylor, 1977).

Convenient methods for the isotopic estimation of DNA synthesis in bulk cultures and for autoradiography have not been previously available for *S. pombe*. It is not possible to label DNA specifically with radioactive thymidine as fungi lack thymidine kinase activity (Grivell & Jackson, 1968). Methods are available in *Saccharomyces cerivisiae* for isotopic detection of DNA by labelling total nucleic acid with a less specific precursor such as uracil or adenine, and then eliminating the radioactivity due to the RNA (Williamson, 1965; Hartwell, 1967; Hatzfield, 1973). In this paper we describe modifications of these methods that will work in *S. pombe*. The method for estimating DNA synthesis in bulk cultures has been used in synchronous cultures to determine the timing of *S*-phase, whilst the autoradiographic method has been used to determine the duration of *S*-phase. With some of the strains the autoradiographic method could also be used to determine the timing of *S*-phase and the variability in the length of the G_1 phase in sister cells.

Our results support the hypothesis that the initiation of *S*-phase is subject to the 2 controls described above, and that there is a cross-over from one control to the other at a particular cell mass at division. We also found that the duration of *S*-phase is short, being only about 0·1 of a cell cycle, and showed no consistent relationship with varying cell mass at *S*-phase. Variability in the length of G_1 of sister cells was found to be very small compared with the sister-sister variation in total cell cycle time, suggesting that G_1 duration and *S*-phase initiation are under precise control.

MATERIALS AND METHODS

Strains, media and growth conditions

Wild type 972 and 4 mutant strains *wee* 1.1, *wee* 1.50, *wee* 2.1 and *cdc* 2.M35 were used (Nurse, 1975; Thuriaux *et al.* 1978; Nurse & Thuriaux unpublished). Cells were routinely grown at 25 °C with shaking in a minimal medium described by Nurse *et al.* (1976).

Preparation of synchronous cultures

Synchronous cultures were prepared by selecting slowly sedimenting small cells after centrifugation in lactose gradients. The cells of the parent asynchronous culture were collected by filtration, suspended in a small volume of medium and centrifuged in 7·5–30% lactose gradients (made up in medium). Small synchronous cultures, as used in DNA labelling experiments, were prepared using tubes as described by Mitchison & Carter (1975). Large synchronous

cultures, as used for measuring bulk DNA, were prepared using a zonal rotor (MSE type A), as described by Mitchison & Carter (1975). In both cases, a fraction of small cells was collected after centrifugation and innoculated into fresh medium.

Measurement of cell number, DNA and protein content of cells

These were performed as described by Nurse *et al.* (1976).

DNA labelling and its measurement

DNA was labelled by the addition of [6-^{3}H]uracil (20 Ci/mmol, Amersham) to cell cultures, followed by incubation for varying periods of time. Pulse labels were terminated by transferring samples to a CO_2/ethanol bath followed by storage at -20 °C. Incorporation of radioactivity into DNA was measured by making up samples to 0·5 M NaOH, 10 mM ethylenediaminetetra-acetic acid, 100 μg/ml calf thymus DNA and incubating at 40 °C for 90 min. Samples were then cooled to 0 °C and acidified by addition of 60 % perchloric acid (PCA). Precipitation was allowed to proceed for at least 1 h at 0 °C, after which the samples were centrifuged at 5000 g for 2·5 min, and the pellet washed once in 0·5 M PCA containing 100 μg/ml uracil and twice in 70 % ethanol, 0·1 M NaCl (both at 0 °C). The pellets were then dried under vacuum and hydrolysed in 0·5 ml 0·5 M PCA for 30 min at 70 °C. The hydrolysate was centrifuged for 1 min at 5000 g and 0·4 ml of the supernatant was added to 10 ml of scintillant containing 60 % toluene, 40 % methoxyethanol, 0·5 % butyl PBD. The samples were counted in a Packard Tricarb scintillation spectrometer.

Chromatography of HCl digests of the PCA hydrolysate (Fig. 1, p. 218) showed that no radioactivity remained as uracil and that at least 90 % of the radioactivity was in the form of cytosine and thymine. At least 90 % of the radioactivity of the PCA hydrolysate was shown to be DNase digestible when the final pellet was incubated in 50 mM 2(*N*-morpholino)ethane sulphonic acid, 2·5 mM $MgCl_2$ buffer pH 7·0 containing 100 μg/ml DNase. Under conditions of long-term labelling about 1 % of the total incorporated radioactivity was in the form of DNA.

The visualization of S-phase by DNA autoradiography

Cells were grown in minimal medium and labelled for 10–40 min in 50 μCi/ml [8-^{3}H]guanosine (10 Ci/mmol, Amersham). Pulse labels were terminated by chilling the cells to 0 °C and adding an equal volume of cold (0 °C) 0·5 M PCA. The cells could be left at this stage for several hours if required. Normally, cells were left at 0° for 5 min, collected by centrifugation, resuspended in 0·5 M PCA, and left at 0 °C for 15–30 min. The cells were then collected by centrifugation, washed twice in distilled water, suspended in 0·3 M sodium chloride, 0·03 M sodium citrate buffer pH 7·0 containing 200 μg/ml boiled RNase A (Sigma), and incubated at 37 °C for 70 min (with intermittent mixing). After this RNase digestion, which renders at least 99·3 % of the total incorporation acid-soluble, the cells were collected by centrifugation, washed twice with 100 μg/ml guanosine, suspended in 0·4 % formaldehyde (0·1 M phosphate buffer pH 7·0), and left at room temperature for 30 min. The cells were then collected by centrifugation, washed once with distilled water, suspended in 0·2 M NaOH at 25 °C for 100 min, and finally washed 3 times with distilled water. Aliquots of the resulting cell suspension were dried directly onto microscope slides rubbed with a thin film of gelatin, and were then dipped in photographic emulsion and exposed at 4 °C. Ilford nuclear research emulsion (in gel form; type L4 or K2) was used; it was heated for 20–30 min at 45 °C prior to dipping of slides. The exposed autoradiographs were developed for 5 min in 1:2 D19 developer (Kodak) at 20 °C and fixed for approx. 3 min in Acufix. The slides were then washed for 30–60 min in gently running tapwater, stained for 3 min in 0·05 % basic fuschin, and destained for 1·5 min in 30 % ethanol. The autoradiographs were viewed using a 100× objective on a Zeiss photomicroscope.

Chromatography of HCl digests of the PCA hydrolysate of the DNA assay

Five millilitres of 972h$^-$ cells growing at 32 °C in minimal medium (5×10^6 cells/ml) were incubated with 50 μCi of [5,6-^{3}H]uracil for 2·5 h. The cells were then harvested, washed in

0·1 % NaCl containing 100 μg/ml uracil, and then taken through the DNA assay treatment. Volumes of 0·05 ml of the final PCA hydrolysate were made to 6 M HCl, to a final volume of 0·1 ml and digested at 120 °C for 2 h in sealed glass tubes, after which the samples were cooled, the tubes broken, and freeze-dried. The residue was dissolved in 0·02 ml distilled water, loaded on to a thin layer PEI cellulose chromotography plate (Polygram cel 300 PEI), and run in a solvent of 86/14 butanol/water for 4·5 h. The chromatographed strip was cut into 0·5-cm lengths, which were placed in scintillation vials, eluted with 0·5 ml of 0·1 M HCl, 0·2 M KCl, and counted in 5 ml of a scintillant containing 2/1 toluene/Triton X-100 with 0·5 % butyl PBD.

RESULTS

The labelling of DNA with uracil isotopes and its measurement

There are several published methods for the assay of isotopic incorporation into DNA for the budding yeast *S. cerevisiae*, but they either do not work for *S. pombe* (e.g. the method described by Hartwell (1967)), or are too laborious for most purposes (e.g. the method described by Hatzfield (1973)). The details of the DNA assay developed here is described in Materials and methods. Uracil isotopes were used because their uptake and incorporation into DNA is adequate, because all RNA incorporation is alkali-digestible (see Fig. 1), and because of the availability of isotopes that are stable in alkali. Briefly, labelled cells are digested with alkali, acid-precipitated, washed,

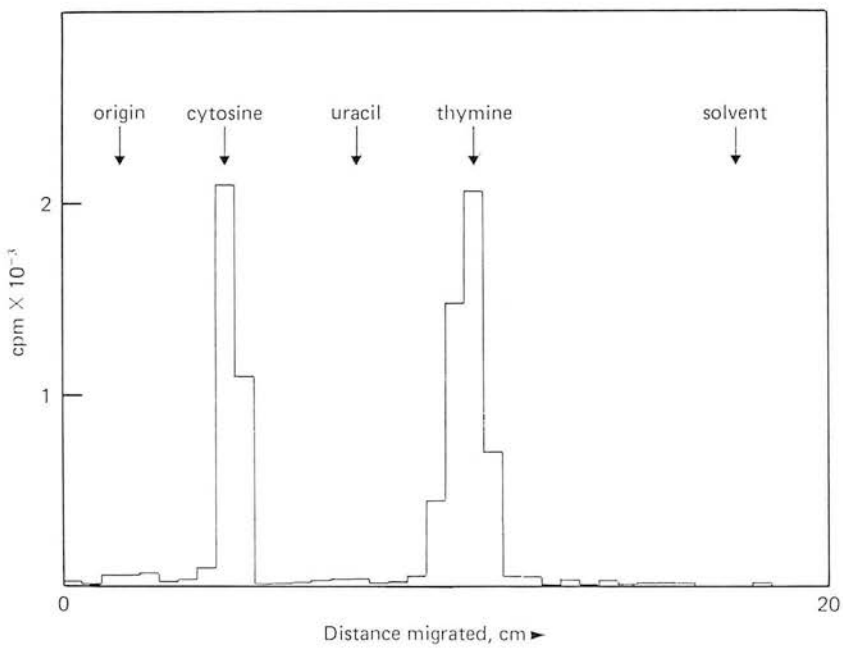

Fig. 1. Chromatography of HCl digests of the PCA hydrolysate of the DNA assay. Wild type 972 h^- cells were labelled with [6-^{3}H]uracil for 1 generation of growth. The cells were harvested, washed, taken through the DNA assay treatment and the final PCA hydrolysate digested with 6 M HCl and chromatographed as described in the Materials and methods. The amounts of label in the chromatogram corresponding to cytosine, uracil and thymine are plotted.

and isotope incorporated into DNA is released by a hot PCA hydrolysis and is counted. At least 90% of the isotope detected by this assay is lost if the alkali-resistant, acid-precipitated pellet (prior to acid hydrolysis) is digested with DNase I. In addition, chromatography of HCl digests of the final PCA hydrolysate (Fig. 1) shows that nearly all the radioactivity residues are cytosine and thymine (as would be expected if all radioactivity is from DNA), and that virtually none is detectable as uracil.

Fig. 2 shows that the pattern of [^{3}H]uracil incorporation into DNA as measured by

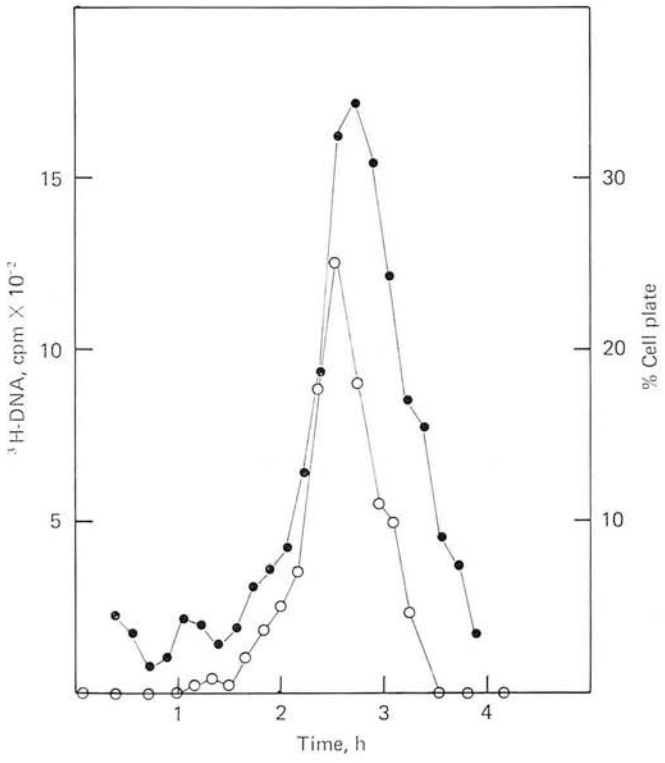

Fig. 2. [6-^{3}H]uracil incorporation into DNA during a synchronous culture. A synchronous culture of 972h$^-$ cells was prepared as described in Methods. The culture was incubated at 32 °C in minimal medium (2×10^6 cells/ml). Every 10 min, 0·3 ml of cells were incubated for 20 min with 10 μCi of [6-^{3}H]uracil (20 Ci/mmol), and taken through the DNA assay treatment. The time of each pulse is taken to be 14 min after its initiation. This is the $t_{0.5}$ for incorporation during a 20-min pulse (see Fig. 3). ●, DNA radioactivity; ○, cell plate index (the % of cells with a septum).

the assay during a synchronous culture, is consistent with previous estimates of the timing of *S*-phase in *S. pombe* (Mitchison & Creanor, 1971). The level of incorporation by G_2 cells is very low, and may be due to mitochondrial DNA replication.

Uracil is not rapidly incorporated into DNA during a pulse and it is difficult to chase (Fig. 3). The latter is probably because at least 99% of the incorporation is in the form of RNA, some of which will be rapidly turned over. After long-term labelling the proportion of the total incorporation as DNA reaches about 1%.

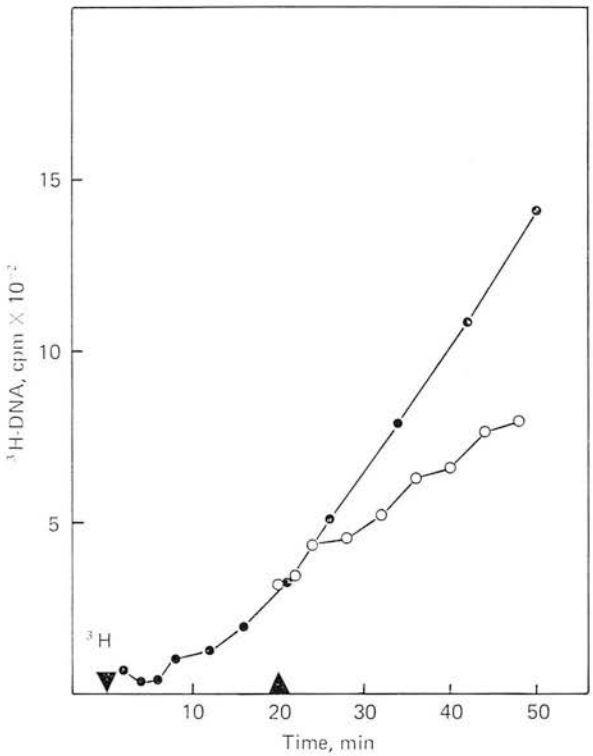

Fig. 3. Kinetics of [6-^{3}H]uracil incorporation into DNA. 972h$^-$ cells were grown at 32 °C in minimal medium (3×10^6 cells/ml.) At the indicated time 200 μCi of [6-^{3}H]uracil (20 Ci/mmol) were added to 6·6 ml of cells. ●, DNA radioactivity in 0·3-ml samples. At t = 20 min, the culture was split and to one half uracil was added to a concentration of 10 mM. ○, chased DNA incorporation.

The visualization of S-phase by DNA autoradiography

Williamson (1965) has developed a method for detecting DNA specific radioactivity within intact cells of the budding yeast *S. cerevisiae*. Cells are pulse labelled with [^{3}H]adenine and incorporation into RNA is selectively removed by a combination of RNase and alkali treatment before they are autoradiographed. The method presented here for *S. pombe* is an adaption of Williamson's method. The principle differences are 2-fold. (1) [8-^{3}H]adenine is replaced by [8-^{3}H]guanosine or, sometimes, [6-^{3}H]-uracil. A significant proportion of adenine incorporation into non-DNA material is resistant to alkaline hydrolysis. (2) The strong alkali treatment during the final stage of Williamson's method is replaced by a much milder treatment. The 1 M NaOH used by Williamson (1965) completely lyses cells of *S. pombe*.

The details of the DNA autoradiography method are given in Materials and methods. Cells and their nuclei remain fully intact after the treatment if grown in minimal medium as a batch culture. However, this is not true for all types of cell. For instance, the nuclei of cells grown under nitrogen limitation in the chemostat are partially destroyed by the terminal alkali step. Therefore, this can be omitted when such cells are prepared for autoradiography.

The major criteria used in evaluating the success of the method were that the label be localized over the nuclei, and that only those cells of the population that were expected to have been in *S*-phase during the pulse were labelled. Mitchison & Creanor (1971) determined the position of *S*-phase in *S. pombe* at around septation by measuring the DNA content of synchronous cultures. The stage of a cell in the cell cycle is easily derived in *S. pombe* from its length (Mitchison, 1970); the cells are rod-shaped and extend only in length as they grow. There is a brief constant-length stage while the cell is laying down and completing its septum. So it is expected that nuclei labelled during a short pulse will be in cells with septa or in short cells which have just completed division.

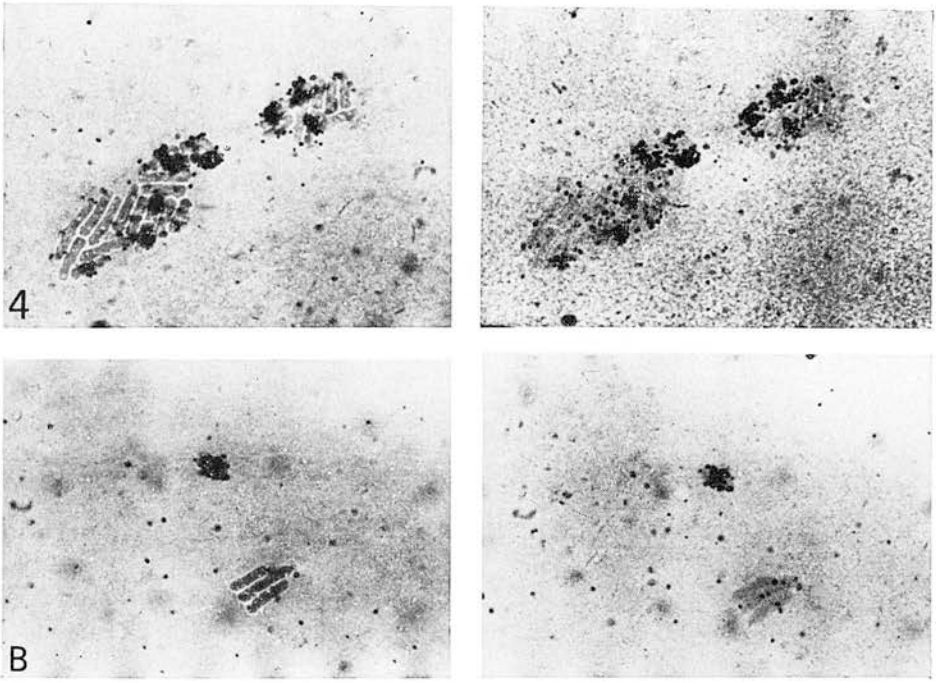

Fig. 4. DNA-specific autoradiographs of pulse-labelled *S. pombe*. 972h$^-$ cells growing in minimal medium at 32 °C (3×10^6 cells/ml) were pulse labelled for 20 min with 50 μCi/ml [8-^{3}H]guanosine. The cells were then taken through the DNA autoradiograph treatment, fixed onto slides, and autoradiographed as described in Methods. They were exposed for 3–4 weeks. The photographs on the left are focused on the cells, whereas those on the right are focused on the grains in the emulsion over the cells.

This was found to be the case as only cells with septa and some small cells were densely labelled after a 20-min pulse label at 32 °C. Moreover, the label in such cells is almost always localized over the nucleus (Fig. 4). The distribution of radioactivity amongst cells at different stages of the cycle was first analysed by grain counting. Fig. 5 shows the distribution of grain counts after a low exposure time amongst cells of different ages (as judged by cell length and presence of a septum). However, grain

counting is difficult to perform satisfactorily when the grains are densely localized. Therefore, a quantitative measure of the cell cycle specificity of labelling was also obtained by pulse labelling successive samples from a synchronous culture, treating the cells from each sample as if for autoradiography, and counting the residual cellular radioactivity on filters in a scintillation spectrometer, instead of fixing the cells on slides and autoradiographing them (Fig. 6). Data from this experiment may be used

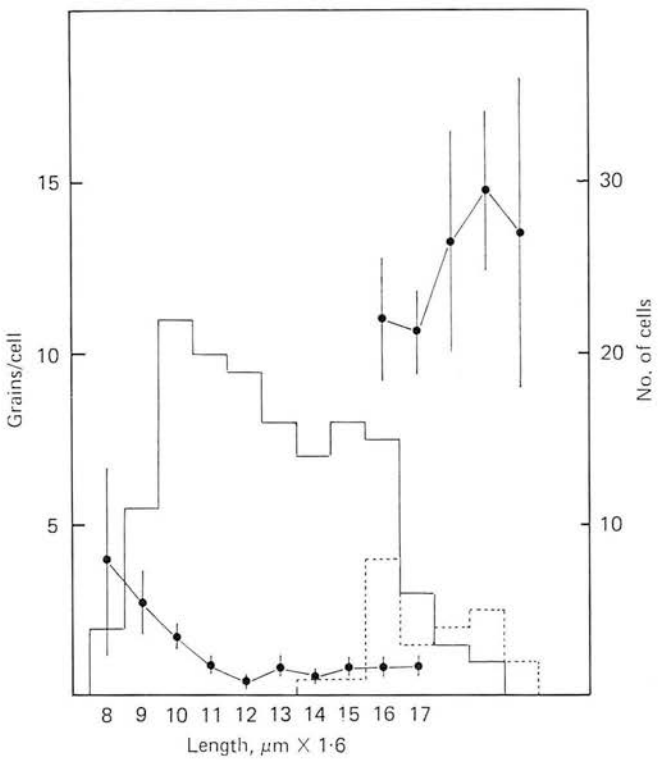

Fig. 5. The distribution of DNA-specific grain counts during the cell cycle. 972h⁻ cells were labelled and treated for DNA specific *in situ* autoradiography. The autoradiographs were exposed for only 5 days (usually a month is required for dense labelling of nuclei). The mean and standard error of the number of grains per cell are plotted against cell length. The size distribution of the population is also presented. The data on the grains per cell in cells containing a septum are presented separately. The size distribution of such cells is given by the 'dotted line' histogram. The grains per cell for this distribution are plotted on the same scale as the rest of the cells; the points are all in the right-hand top corner of the graph.

to estimate the proportion of residual radioactivity left after the treatment, that is in nuclear DNA. This is done by measuring the proportion of the cpm which is exclusively under the, presumably, *S*-phase peak in Fig. 6. The value is 79%. Therefore, the residual radioactivity, over a complete cell cycle, accounts for only 21%. This residual will partly consist of mitochondrial DNA synthesis (probably 5%, according to Bostock (1970)) and some nuclear DNA synthesis due to lack of synchrony. Consequently, the amount of non-DNA radioactivity left after the

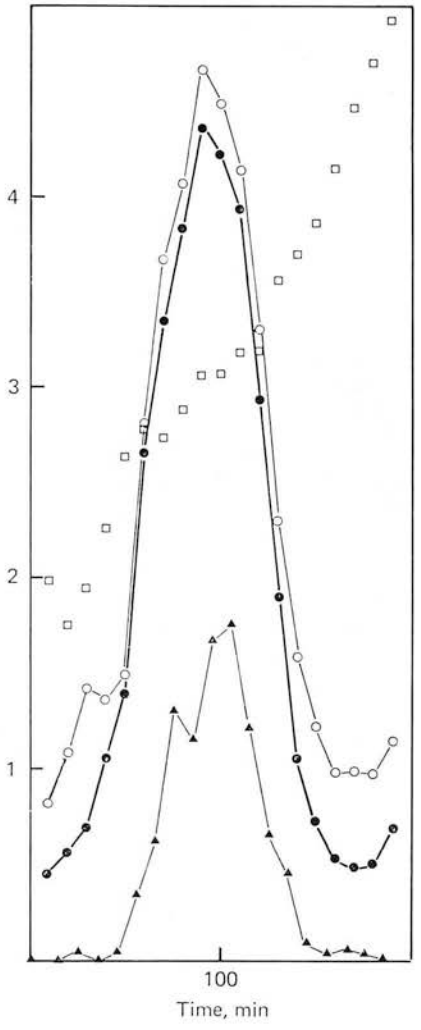

Fig. 6. The rate of incorporation of [8-^{3}H]guanosine into DNA as measured by the autoradiographic method during a synchronous culture. A synchronous culture of 972h$^-$ cells was prepared as described in Methods. The cells were incubated at 32 °C in minimal medium (3×10^6 cells/ml). At 10-min intervals, a 3-ml sample of cells was incubated with 10 μCi [8-^{3}H]guanosine (10 Ci/mmol) for 20 min. The sample was then chilled, a 0·1-ml aliquot was taken for uptake measurement, an equal volume of ice-cold 0·5 M PCA was added, and the sample was left at 0 °C until all the samples were ready for processing. The samples were then taken through the DNA-specific autoradiograph treatment as far as the formaldehyde fixation step. A sample of the cells were collected on a GFA filter (washing with distilled water containing 100 μg/ml guanosine). The dried filters were counted in a toluene butyl PBD scintillant. These samples gave the RNase-resistant radioactivity. The rest of the cells were treated with 0·2 M NaOH as described in Methods before being similarly collected and counted. These samples gave the residual radioactivity after the full autoradiograph treatment. Both sets of results are plotted as a percentage of the uptake per unit volume. They are plotted on an arbitrary linear scale whose unit relation to real values is given within the brackets of the symbol key. □, uptake during the pulse/0·1 ml (5×10^3 cpm); ●, residual cpm left after full autoradiograph treatment as a % of uptake per unit volume (0·1 %); ▲, cell plate index (10 %); ○, RNase-resistant cpm as a % uptake per unit volume (0·1 %).

autoradiograph treatment may be less than 16%. The proportion of radioactivity left after the treatment that is DNase-digestible has not been determined. This enzyme, unlike RNase either does not enter PCA-treated cells or does not digest their DNA. However, it is possible to determine what proportion of the residual radioactivity is released after a 90 °C TCA hydrolysis. This treatment will probably only release radioactivity of nucleic acid origin. It is found that only 10% of the radioactivity is resistant to a 15-min 10% TCA treatment at 90 °C. This suggests that 90% of the cpm left after the autoradiography treatment is in the form of DNA, though it is possible that some is in RNA as it also will be susceptible to the acid hydrolysis.

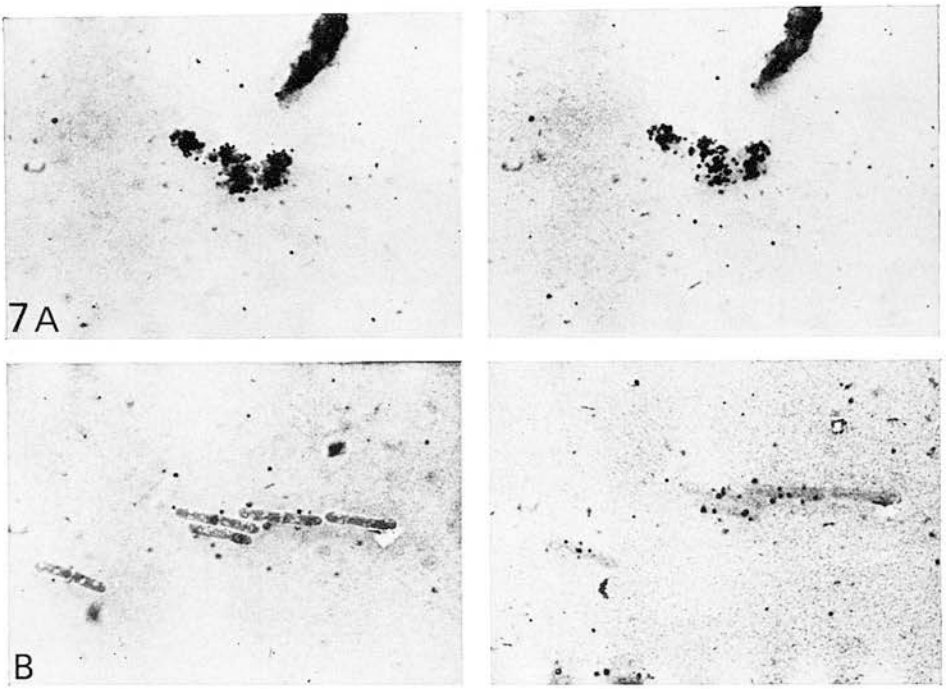

Fig. 7. A comparison between 972h⁻ and *cdc* 10–129h⁻ in their pattern of nuclear labelling on shift to 36 °C. 972h⁻ and *cdc* 10–129h⁻ were grown at 25 °C in minimal medium to 4×10^6 cells/ml. Both cultures were then shifted to 36 °C and pulse labelled for 20 min with 65 μCi/ml [8-^{3}H]guanosine (10 Ci/mmol) after 150 min at 36 °C. Both sets of cells were then treated for DNA-specific autoradiography. Exposure was for 1 month. A, 972h⁻ cells; B, *cdc* 10–129h⁻ cells. The photographs on the left are focused on the cells, whereas those on the right are focused on the grains in the emulsion over the cells.

Fig. 6 also demonstrates that whilst the RNase removes most of the radioactivity incorporated outside the *S*-phase, the terminal alkali treatment does serve to reduce further the presumably non-DNA background. The radioactivity left after the complete treatment is 0·2% of the uptake of the pulse per unit volume (averaged over a complete cell cycle).

The experiment illustrated in Fig. 7 shows that if cells are prevented from entering *S*-phase, then those nuclei which would normally be labelled are no longer so. In this experiment 972 h⁻ and *cdc* 10-129 h⁻ cells (the latter is a ts mutant defective in

the initiation of DNA synthesis; Nurse *et al.* 1976) were shifted to the restrictive temperature and pulse labelled 150 min after the shift, at which time both wild type and mutant cell populations still have a similar proportion of cells with septa which normally contain labelled nuclei. In this instance, such septated cell nuclei from *cdc* 10-129 h$^-$ cells will not be undergoing DNA synthesis and, as expected, they are not labelled. None of the *cdc* 10-129 h$^-$ septated cells had labelled nuclei, whereas 87% of the 972 h$^-$ septated cells had.

The DNA-autoradiography method can be varied for cells growing in different physiological conditions. [6-^{3}H]uracil can also be used as a label, though the background is slightly higher. For cells growing in batch minimal medium, the specificity of [^{3}H]guanosine labelling is superior. However, the latter is not satisfactory for cells grown under nitrogen limitation in the chemostat and [^{3}H]uracil is superior under these conditions.

The standard method of analysis of autoradiographed cells involves an exposure until the radioactive nuclei are densely labelled. Cells in *S*-phase can then be scored unambiguously without grain counting.

Timing of S-phase in the cell cycle

To examine the effect that cell mass has on the timing of *S*-phase, we determined when it takes place in strains which vary in cell mass at division over a 3-fold range. The 5 strains grown at 25 °C have similar generation times, but vary in their cell length at division and in their mean protein content per cell (Table 1). Two methods have been used to establish the timing of *S*-phase in the cell cycle: measurement of DNA during synchronous culture and autoradiography.

Selection synchronous cultures were prepared from the 5 strains and DNA was monitored either by estimation with diphenylamine or by the isotope method described above. When tested on the same cultures the 2 methods gave similar results. The timing of the mid-point in DNA replication with respect to the end of mitosis is given in Table 2. In the strains with the larger cell masses, *cdc* 2.M35 and 972 wild type, the mid-point is about 0·1 of a cell cycle after mitosis. This interval increases as cells get smaller and attains 0·5 of a cell cycle in the strain with the smallest cells, *wee* 1.1. The protein content per cell at the midpoint of DNA replication is similar in the smaller strains *wee* 1.1 and *wee* 2.1 but gradually increases in the larger strains (Table 2). These results are in good agreement with the hypothesis that the initiation of DNA replication is subject to the 2 controls. In cells dividing at a small mass such as *wee* 1.1 and *wee* 2.1, there is a long G_1, and *S*-phase is initiated when cells attain a mass equivalent to 7–7·5 pg protein/cell. In the larger cells such as *cdc* 2.M35 and 972 wild type, cells are always above this cell mass and the G_1 is constant and short. In these cells *S*-phase is initiated when mitosis and a minimum time in G_1 have been completed.

The minimum time in G_1 can be approximately estimated from the results with *cdc* 2.M35 and 972 wild type. The time from the end of mitosis to the midpoint of DNA replication is 0·17 and 0·16 respectively. These values are clearly overestimates as they are calculated from the end of mitosis to the midpoint of DNA replication

Table 1. *Data relating to the cell cycles of the 5 strains of S. pombe investigated*

Strain	Generation time, min	Cell length at division, μm	Mean protein content per cell, pg	% cells septated	% cells binucleated	% cells labelled	% septated cells labelled	% septated cells asymmetrically labelled
wee 1.1	270	8·6	7·8	14	5	21·1	0	—
wee 2.1	250	9·2	8·4	14	4	20·0	4	—
wee 1.50	240	10·9	9·7	13	5	28·3	37	4·8
972	230	12·9	12·0	9	4	27·2	74	2·7
cdc 2.M35	270	22·4	20·4	8	5	22·5	73	3·0

* Counting septated cells as two.

Table 2. *Length of G_1 and S-phase and cell mass at S-phase*

Strain	Timing from cell separation to mid-point of *S*-phase*	Timing from end of mitosis to initiation of *S*-phase (G_1) from synchronous cultures†	Timing from end of mitosis to initiation of *S*-phase (G_1) from autoradiographs	Duration of *S*-phase	Protein content per cell at mid-point of *S*-phase, pg/cell‡
wee 1.1	0·31	0·51	—	0·11	7·8
wee 2.1	0·15	0·36	—	0·07	7·4
wee 1.50	0·05	0·23	0·17	0·12	8·1
972	−0·02	0·12	0·08	0·09	9·2
cdc 2.M35	−0·01	0·13	0·09	0·07	16·1

* Derived from synchronous cultures.
† Derived from column 1 correcting for % binucleated and septated cells in an asynchronous population and the duration of *S*-phase.
‡ Derived from synchronous cultures and mean protein content per cell in an asynchronous population.

rather than to its initiation. Correcting for the duration of *S*-phase the values are 0·13 and 0·12 (Table 2). A better estimate can be made by autoradiography. The 5 strains were labelled for 30 min, equivalent to 0·11–0·13 of a cell cycle. In *cdc* 2.M35 and 972 wild type, only cells with septa and small cells at the very beginning of the cell cycle were labelled. This result is consistent with the mid-point of DNA replication taking place 0·1 cell cycle after mitosis at around cell septation. The G_1 period can be estimated from the fraction of cells with a septum which are unlabelled, since these cells will not yet have initiated *S*-phase. These values are given in Table 1 and after adding the fraction of binucleates and correcting for the cell age distribution, the value of the minimum time in G_1 is 0·09 for *cdc* 2.M35 and 0·08 for 972 wild type. These values may be overestimates if the rate of synthesis of DNA at the very beginning of *S*-phase is too low to detect, or if it is difficult to label the early part of *S*-phase due to a lag in the precursor kinetics. Both of these problems have been minimized by using isotope of high specific activity and long exposure times so that cells were either strongly labelled or not labelled at all.

The fraction of cells with a septum which are unlabelled was also counted in the 3 strains with smaller cells. As cell mass at division decreased the fraction of unlabelled septated cells increased until in *wee* 1.1 none at all were labelled (Table 1). This result is quite consistent with the G_1 period increasing in length as cell mass at division becomes smaller, as found in the synchronous culture.

The autoradiographs can also be used to determine how synchronously *S*-phase is initiated in 2 sister cells. In *cdc* 2.M35, 972 wild type and *wee* 1–50, DNA replication is initiated during the septated stage of the cell cycle. Thus, asynchrony of initiation between 2 sisters can be detected by observing the number of septated cells in which only one sister is labelled. As can be seen in Table 1, the fraction of septated cells which are asymmetrically labelled is very low for all 3 strains, indicating that initiation is almost simultaneous in sister cells.

The mean difference in time of initiation between sisters can be estimated from the proportion of asymmetrically labelled pairs of nuclei in septated cells in the total cell population. Asymmetrically labelled daughter nuclei may be due either to pairs in which only one daughter has initiated by the end of the pulse, or to pairs in which one daughter had terminated *S*-phase by the beginning of the pulse and the other had not terminated *S*-phase by the end of the pulse. However, given the limited duration of the septated period and the relatively long duration of the pulse labels used here, the latter type are unlikely to occur. Therefore, asymmetrically labelled pairs of nuclei must represent all those sister pairs of the population in which only one sister has initiated at any given instant (in this case, at the end of the pulse). Correcting for the age distribution of an asynchronous population, this proportion of sister nuclei pairs in which only one sister has initiated *S*-phase (at any given instant) must represent the mean difference between sisters in their time of initiation. The mean differences for *wee* 1.50, wild type 972, and *cdc* 2.M35 are extremely small, ranging from 0·003 to 0·009 of a cell cycle or 0·8 to 2·2 min. This is far smaller than the mean difference between daughters for their total cell cycle time, which is about 0·1 of a cell cycle (derived from Fantes, 1977).

The duration of S-phase in S. pombe

The length of *S*-phase can be estimated from a knowledge of the proportion of cells of a steady-state asynchronous population that are in the process of DNA synthesis. Individual cells in the process of DNA synthesis can be detected by autoradiography of pulse-labelled cells. If the pulse length is very short in comparison to the length of *S*-phase, then the duration of *S*-phase, in terms of a fraction of the cell cycle, is directly given by the proportion, corrected for the age distribution, of cells whose nuclei are labelled. In this instance, the density of labelling of different *S*-phase nuclei will be as uniform as the rate of DNA synthesis during *S*-phase, assuming that all stages of *S*-phase are labelled with equal specific activity of tracer. If the rate of DNA synthesis is fairly constant during *S*-phase, then the scoring of labelled cells will be unambiguous.

Unfortunately in order to avoid excessively long exposure times, we have been forced to use pulse labels whose duration is by no means short in comparison to the length of *S*-phase. In this case, theoretically, the proportion of labelled cells, correcting for the age distribution, will be determined by the combined length of the pulse label and *S*-phase (in terms of a fraction of a cell cycle). In practise, this introduces 2 potential complications.

The first is that, since the length of the pulse label becomes an important parameter in the estimation, the kinetics of the pulse label also become important. For instance, if virtually no label is incorporated into DNA in the first 5 min of the pulse, then the effective length of a 20-min pulse will be only 15 min.

The second is that the level of labelling amongst different cells will no longer be as uniform as the rate of DNA synthesis during *S*-phase, since a significant proportion of labelled cells will only have been in *S*-phase for a small fraction of the pulse label. This means that some cells may be much less heavily labelled than others and may thus be missed in the scoring.

The extent of the first problem was investigated by analysing the kinetics of [^{3}H]guanosine incorporation into DNA. Wild-type 972 cells were pulse labelled for various lengths of time, treated for DNA autoradiography, and the quantity of isotope remaining counted. As can be seen in Fig. 8, there is some curvature in the incorporation of label, indicating that the precursor pools are not fully labelled until about 20 min after the beginning of the pulse. However, it is clear that incorporation does occur continuously from the beginning of the pulse label. Moreover, the rate during the first 10 min is at least 0·4 of that between 30 and 40 min. Therefore, as long as the autoradiographs are sufficiently heavily exposed, there is little danger that the effective length of the pulse label will be significantly less than the period for which the cells are exposed to isotope.

The second problem of non-uniform cell labelling can also be overcome by sufficiently heavy exposure of the autoradiographs. In fact, this problem probably never seriously arises because we have never encountered difficulties in deciding whether or not a cell is labelled. However, since the problem, if it does occur, must be less acute the shorter the pulse label, we have estimated the length of *S*-phase from 4 pulse labels of different lengths.

A formula relating the length of *S*-phase to the proportion of labelled cells after a pulse of given duration is derived in the Appendix. The mid-point of *S*-phase must also be known and this is taken from the values determined in the synchronous cultures (Table 2). The lengths of *S*-phase in wild type 972 have been calculated from pulses of 10, 20, 30 and 40 min and are plotted on Fig. 8. The value estimated from each is identical, suggesting that the practical problems of using finite pulse lengths are not critical.

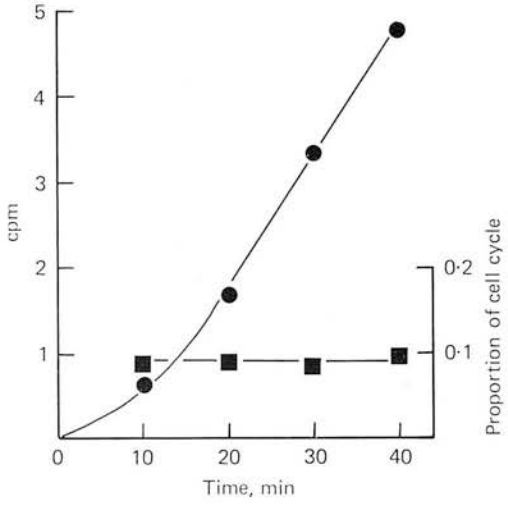

Fig. 8. Time course of the rate of incorporation of [8-^{3}H]guanosine into DNA as measured by the autoradiographic method and calculated length of *S*-phase. An exponentially growing 972h$^-$ culture (3×10^6 cells/ml) labelled and treated as described in Fig. 6. The label incorporated into DNA per 10^3 cells is plotted against the time of the pulse. The calculated lengths of *S*-phase, as described in the Results and Appendix, are also plotted for each time point of the pulse. ●, label in DNA; ■, length of *S*-phase.

The duration of *S*-phase in the 5 strains of different cell masses was determined using a 30-min pulse label. It can be seen from Table 2 that the duration of *S*-phase was very short for all the strains, ranging between 0·07 and 0·12 of a cell cycle. There was no consistent relationship between cell mass at division and the duration of *S*-phase. The 2 strains with mutated *wee* 1 genes had a rather longer *S*-phase than the other strains which might indicate that the *wee* 1 mutations have pleiotropic effects over the duration of *S*-phase.

DISCUSSION

Three problems have been considered in this paper: the relationships between cell mass and the durations of the G_1 and of the *S*-phases of the cell cycle, and the variability in the duration of the G_1 phase. We shall consider each of these problems in turn.

Duration of G_1

The results concerning the relationship between cell mass at division and the duration of G_1 are summarized on Fig. 9. The G_1 phase is longest in cells with the smallest mass at division. As cell mass at division increases, the G_1 phase gradually gets shorter until it reaches about 0·1 of a cell cycle. Beyond this point further increase in cell mass at division does not make the G_1 phase any shorter. Cell mass at S-phase is greatest in cells with the largest mass at division, and both decrease together as far as a cell mass at division of 15 pg protein (Fig. 9).

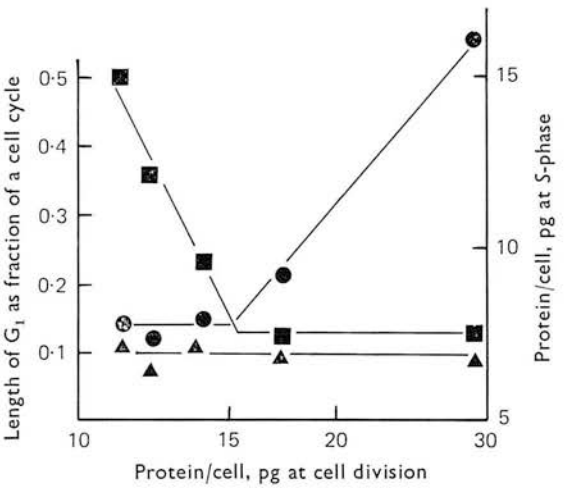

Fig. 9. Length of G_1 and S-phase, and protein content per cell at S-phase against protein content per cell at division. The length of G_1 is taken from the synchronous cultures. The length of S-phase is taken from the autoradiographs. ■, length of G_1; ▲, length of S-phase; ●, protein content per cell at S-phase.

However, cell mass at S-phase does not fall below a value of about 7·5 pg protein per cell, even though cell mass at division continues to fall from about 15 to 11 pg protein per cell (Fig. 9). This is exactly what would be predicted from the hypothesis that the initiation of S is subject to 2 controls: attainment of a critical cell mass and dependency upon completion of the previous mitosis coupled with a short minimum time in G_1. In cells undergoing cell division at a small mass the former control determines the timing of S-phase, which consequently always occurs at a constant cell mass. In cells undergoing cell division at a larger mass the latter control determines the timing of S-phase, and the duration of G_1 is short and constant. The crossover from one control to the other occurs at 15 pg protein per cell at division, the point at which cell mass at the initiation of S-phase ceases to be constant, and where the duration of G_1 begins to be constant. It should be stressed that both controls are operative in both large and small cells but only one of them is important in determining the timing of S-phase in a particular cell.

We have recently discussed in some detail whether the above hypothesis could apply to higher eukaryotic cells (Nurse & Thuriaux, 1977), and refer the interested

reader to this discussion, where we concluded that the hypothesis could act as a useful conceptual framework for considering the varied results obtained with mammalian cells. The mechanism of the 2 controls remains obscure. There are various ways by which a cell may monitor its mass which could be used to define a minimum critical cell mass requirement before *S*-phase can be initiated. Possible mechanisms have been reviewed by Fantes *et al.* (1975).

The dependency of *S*-phase on completion of the previous mitosis and a minimum period of G_1 may be caused by a requirement for reorganization of the chromatin between mitosis and DNA replication. It has been suggested that chromosomes have to decondense after mitosis before *S*-phase can begin in mammalian cells (Rao, Wilson & Puck, 1977), and some changes in chromosome folding between the G_2 and G_1 phases have also been observed in yeast (Pinon & Salts, 1977). Thus the minimum period in G_1 may be because a *S. pombe* cell takes about 0·1 of a cell cycle to decondense its chromosomes sufficiently for DNA replication to be initiated.

Duration of S-phase

There is some evidence that the duration of *S*-phase can be affected by cell mass. Early embryonic *Triturus vulgaris* (Callan, 1973) and *Drosophila melanogaster* (Blumenthal *et al.* 1973) cells have a much shorter *S*-phase than somatic cells, and are of course much larger. The inter-origin sites are much shorter in the embryonic cells indicating that more replicons are switched on in these cells. Similarly, treatment of Chinese hamster mammalian cells with FdU, which allows cell growth to continue in the absence of DNA synthesis, results in cells, which when released from the FdU block initiate more replicons than normal (Taylor, 1977). More directly, it has been shown in *Escherichia coli*, that enlarging the cell will switch on an incorporated F factor replicon, which is normally dormant (Chandler, Silver, Roth & Caro, 1976). All these data could be interpreted in terms of a cell mass control over the number of replicons active in a cell. The bigger the cell, more replicons would be active and *S*-phase shorter. However, in our experiments with *S. pombe* no consistent relationship was observed between cell mass at mitosis and the duration of *S*-phase (Table 2). Cells over a 3-fold range of cell mass, *cdc* 2.M35, wild type and *wee* 2.1, all had *S*-phases of similar duration. This suggests that at higher cell masses, at least in the range considered here, no new replicons are switched on which dramatically shorten the duration of *S*-phase. Two strains, *wee* 1.1 and *wee* 1.50, appeared to have rather longer *S*-phases. This may be due to possible pleiotropic effects of mutations in *wee* 1 over *S*-phase. *wee* 1 is involved in the control initiating mitosis (Nurse, 1975), but could conceivably be altered in nuclear or chromosome structure, such that *S*-phase takes longer to complete.

Variability in the G_1 phase

The average difference in the duration of the G_1 phase between 2 sister cells was at most about 0·01 of a cell cycle. This is far smaller than the average difference in total cell cycle time between 2 sister cells which is about 0·1 of a cell cycle (derived from Fantes, 1977). Thus most of the variability in cell cycle time is introduced later in the

cell cycle of *S. pombe* than the G_1 phase; this situation is in contrast with many other eukaryotic cells in which most of the variability in cell cycle time is thought to be introduced during G_1 (Prescott, 1976). It is also of interest with respect to the transition probability hypothesis of cell proliferation (Smith & Martin, 1973). In this hypothesis the rate of cell proliferation is determined by a random transition during the cell cycle between an A state and a B phase. Once this transition has taken place little further variability is introduced into the cell cycle as the cell traverses the B phase. The transition from the A state to the B phase is thought to take place during G_1 phase which is the most variable part of the cell cycle in most cells (Smith & Martin, 1974). However, this cannot be the case for *S. pombe*. The distribution of cell cycle times in *S. pombe* can be interpreted in terms of a random transition event determining the rate of cell proliferation (Fantes, 1977). But this transition cannot be taking place in G_1 as there is too little variability in this phase of the cell cycle. If a random transition is taking place it must be in the G_2 phase of the cell cycle. Alternatively, the interpretation of the distribution of cell cycle times in terms of a random transition should be treated with some caution.

We should like to thank Murdoch Mitchison for his encouragement throughout this work, and the S.R.C. and M.R.C. (U.K.) for financial support.

APPENDIX

A means of estimating the length of S-phase from the proportion of cells labelled after a pulse label of given duration.

Let $S = 2.z$ = the duration of S-phase, x = the duration of the pulse label, y = the proportion of cells labelled in an asynchronous culture after a pulse of duration x, r = the mid-point of S-phase

All units are in terms of a fraction of the cell cycle.

If q and p are any 2 stages in the cell cycle ($p > q$), then the proportion of an asynchronous cell population that lies between q and p is:

$$= \frac{\int_q^p 2^{(1-t)}\,dt}{\int_0^1 2^{(1-t)}\,dt}$$

$$= 2^{(1-q)} - 2^{(1-p)}.$$

Now, after a pulse label of duration x, cells between $q = r-z$ and $p = r+z+x$ in the cell cycle will be labelled.

$$y = 2^{(1-r+z)} - 2^{(1-r-x-z)}$$

$$= 2^{(2-r-x}/2).\sinh(\log_e 2\,(z+x/2)).$$

Thus,

$$S = 2z = \frac{2.\sinh^{-1}((y/2)\,(2-r-x/2))-x}{\log_e 2}.$$

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(*Received* 9 *February* 1979)

(*Reprinted from Nature, Vol. 215, No. 5103, p. 873 only, August 19, 1967*)

Effect of Light on Cell Division in Plant Tissue Cultures

LIGHT strongly influences many aspects of growth in plants. There have, however, been few studies on the effects of light on cell division in non-green plant tissue cultures. In the course of investigating the physiology of cell division in developing callus cultures of *Helianthus tuberosus* it has been observed that light can have an inhibitory effect on cell division.

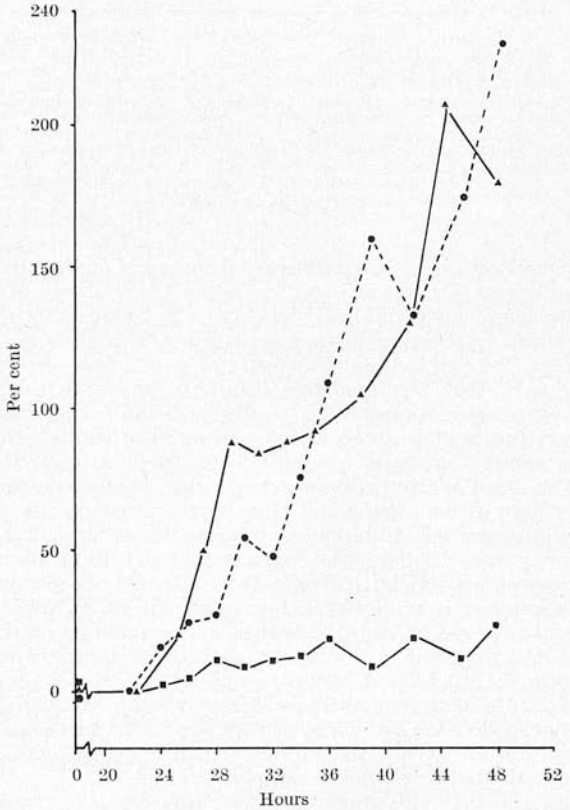

Fig. 1. Percentage increase in cell number per explant with time of incubation. Explants were removed from tubers in green light and cultured in groups of 80 in 12 ml. medium containing 10^{-5} molar 2,4-D, in 9 cm Petri dishes, at 25° C. Agitation was on a reciprocating shaker operating at 50 c/s with a 7 cm displacement. The explants were grown in total darkness (▲), 120 ft.-candles (●) and 450 ft.-candles (■). Both light sources were mixed fluorescent and tungsten. Cell number data were derived from counts on 5 per cent chromic acid macerates of five explant samples.

J. Mol. Biol. (1970) **49**, 681–692

Ribosomal RNA Precursors in Plants

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(*Received 30 June 1969, and in revised form 1 December 1969*)

A high molecular weight, rapidly-labelled ribosomal RNA precursor in the pea-root tip and in artichoke-tuber tissue is described. It has a molecular weight, determined by polyacrylamide-gel electrophoresis, of about $2{\cdot}3 \times 10^6$ daltons. The pea precursor RNA consists of two components which can just be distinguished by gel electrophoresis, whereas that from the artichoke appears homogeneous. $2{\cdot}3 \times 10^6$ daltons RNA is labelled within 15 minutes of incubation in [^{32}P]orthophosphate, and is followed by a second precursor of molecular weight $1{\cdot}4 \times 10^6$ daltons. Also appearing at this time is a third component of $0{\cdot}9 \times 10^6$ daltons. The base composition of the $2{\cdot}3 \times 10^6$ and $1{\cdot}4 \times 10^6$ daltons RNA's is similar to that of ribosomal RNA, in contrast to heterogeneous RNA. In older regions of the pea root, in which the rate of ribosome synthesis is low, the synthesis of precursor molecules is decreased. In excised pea-root tips, cultured *in vitro*, in which there is no net protein or RNA synthesis, label is incorporated into the precursor. Assembly of ribosomes, however, is prevented by inhibition of its processing, with accumulation of the $2{\cdot}3 \times 10^6$, $1{\cdot}4 \times 10^6$ and $0{\cdot}9 \times 10^6$ daltons components. The method of synthesis of ribosomal RNA in plants is discussed in relation to that in animals.

1. Introduction

The mechanism of synthesis and assembly of ribosomes has only been investigated thoroughly in animal cells. In mammalian cells the precursor to ribosomal RNA is synthesized in the nucleolus (Perry, 1962; Scherrer, Latham & Darnell, 1963); it is a molecule of 45 s and has a molecular weight of about $4{\cdot}4 \times 10^6$ daltons (Weinberg, Loening, Willems & Penman, 1967; McConkey & Hopkins, 1969). This molecule is methylated during or immediately after synthesis, and then cleaved in several stages to form the 28 s and 18 s rRNA (Greenberg & Penman, 1966). The molecular weight of the precursor is nearly twice that of the sum of the rRNA components ($1{\cdot}7 \times 10^6$ and $0{\cdot}7 \times 10^6$ daltons). The excess RNA, which is not methylated, is discarded during the process of rRNA formation (Weinberg *et al.*, 1967; Zimmerman & Holler, 1967; Jeantur, Amaldi & Attardi, 1968). Recent experiments have indicated that the 45 s precursor is cleaved, losing non-ribosomal excess RNA, to 41 s RNA; the latter is then split to give the 32 s precursor to 28 s rRNA and the 20 s precursor to 18 s RNA (Weinberg & Penman, 1970). The 32 s RNA has a lifetime of about one hour in the nucleolus while the smaller rRNA component, the 18 s, is formed from 20 s and transported to the cytoplasm rapidly (Girard, Penman & Darnell, 1964).

Less extensive experiments on the rRNA precursors have been described in the newt *Triturus* (Gall, 1966) and in *Xenopus* (Landesman & Gross, 1969; Loening, Jones & Birnstiel, 1969).

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Until recently no such high molecular weight rRNA precursor could be found in plants. The presumed precursor in pea-root tips (Loening, 1967) was found to be labile and frequently lost during preparation of nuclear fractions. A more stable component was found in carrot-disk tissue (Leaver & Key, 1970, accompanying paper) and in artichoke tuber.

In this paper we describe the isolation and properties of the precursor rRNA in pea-root tips and in artichoke-tuber tissue. These two species illustrate different aspects of the properties of the precursor and of rRNA synthesis in plants. The artichoke precursor rRNA seems to be a homogeneous molecule, relatively stable during extraction; a chase incubation after radioactive labelling is effective in this tissue and demonstrates the turnover of the precursor. In pea-root tips, the precursor is less stable during isolation, and consists of at least two components. The root-tip conveniently allows examination of two conditions in which rRNA synthesis is decreased: first, in the older parts of the root where ribosome synthesis progressively decreases but incorporation of isotope continues, and second, in excised, cultured, root tips in which net synthesis of ribosomes and of protein is inhibited. The experiments indicate that in the first situation the synthesis of the precursor is decreased, while in the second the processing of the precursor to rRNA is inhibited.

2. Materials and Methods

(a) *Growth and labelling of pea roots*

Pea seeds (*Pisum sativum* var. Meteor) were sterilized by washing in 90% ethanol followed by 1% sodium hypochlorite, and were rinsed several times in sterile distilled water. They were planted under sterile conditions in moist Vermiculite (2·5 vol. : 1 vol. of water, autoclaved) and grown at 25°C for 2 to 3 days. The seedlings were harvested when the roots were about 4 cm long, and were washed in distilled water at 25°C before transfer to radioactive media.

Intact seedlings were incubated with their root tips dipping in 20 ml. distilled water per 60 seedlings, containing 1 to 2 mc [^{32}P]phosphate (specific activity 30 to 40 c/mg P, obtained from the Radiochemical Centre, Amersham, and dried to remove HCl) at 24 to 25°C. To follow RNA synthesis in different regions of the root, the roots were cut after incubation into 3 segments measuring 1·6 mm (the tip), 1·8 and 3 mm.

For experiments on cultured root tips, the terminal 3 mm were cut in sterile 2% sucrose and ^{32}P (0·25 to 0·5 mc/ml.) was added immediately. 20 to 30 root tips/ml. were incubated at 25°C.

(b) *Growth and labelling of artichoke tubers*

Plants of the Jerusalem artichoke (*Helianthus tuberosus* var. Bunyards Round, a single clone) were grown at the Department. Explants of 9 mg were cut from parenchymal tissue of the tubers and cultured aseptically in liquid medium (4% sucrose, mineral salts (Bonner & Addicott, 1937) and 10^{-5} M-2,4-dichlorophenoxyacetic acid) as described by Fraser, Loening & Yeoman (1967). 30 explants were cultured in 5 ml. medium at 25°C in the dark, for 20 to 40 hr. Most of the undamaged cells of the explants divide synchronously after a culture time of about 25 hr; the time taken for the first division increasing with the age of the tuber. Light inhibits cell division (Fraser *et al.*, 1967) but has little effect on rRNA synthesis. The RNA content of the tissue increased fourfold during the first 50 hr of culture.

Handling of the explants for radioactive incubation was in normal laboratory light; most of the incubation time was in the dark. 30 explants were washed in 10 ml. sterile phosphate-free culture medium to remove excess phosphate. For the pulse incubation the explants were transferred to 3 ml. of phosphate-free medium containing up to 1 mc of ^{32}P. It was found that short periods of exposure of the tissue to phosphate-free medium had no effect on the labelling of the RNA or on the subsequent growth of the explants.

The chase incubation was in 10 ml. of sterile complete culture medium. For these incubations, the explants were in punctured centrifuge tubes which could be readily transferred from one medium to the next.

(c) *Cell fractionation and extraction of RNA*

The homogenization medium for the artichoke tissue contained: 0·3 M-sucrose, 0·05 M-KCl, 1 mM-magnesium acetate, 0·03 M-Tris, adjusted to pH 7·6 at 0°C with HCl; 5 mM-dithiothreitol (Cleland, 1964). 30 explants were homogenized for 20 sec at 0°C using 2 ml. of medium, and were immediately centrifuged at 1000 *g* for 5 min, to give a nuclear-debris fraction and a cytoplasmic supernatant. Detergents for RNA extraction were added immediately after centrifugation.

A high pH and the addition of low-molecular weight yeast RNA was found to be essential for the reproducible isolation of the precursor rRNA from pea-root debris fractions. The medium used contained: 0·5 M-sucrose, 0·05 M-KCl, 5 mM-magnesium acetate, 0·05 M-Tris, adjusted to pH 7·8 at 0°C; 0·25 mg yeast RNA/ml. (B.D.H. or Sigma). The root tips were homogenized in a motor-driven Teflon-in-glass homogenizer, using 1 ml. for 20 tips. Debris and supernatant fractions were separated by centrifugation as for the artichoke tissue.

RNA was extracted from the cell fractions, or by homogenization of the whole tissue, as described previously (Parish & Kirby, 1966; Loening *et al.*, 1969).

(d) *Gel electrophoresis*

Gels containing 2·2 or 2·4% recrystallized acrylamide were prepared as described previously (Loening, 1967,1969).

(e) *Determination of base compositions*

The RNA in selected gel slices was hydrolysed either with 10% (v/v) piperidine containing 1 mM-EDTA at 60°C for 48 hr, or with 0·3 M-KOH at 38°C for 15 hr. Other details were as described previously (Loening, 1967; Loening *et al.*, 1969).

(f) *Determination of molecular weights*

Molecular weights were obtained from the gel electrophoretic-mobility as described by Loening (1969). The molecular weights of the RNA of the plants were previously determined as $1{\cdot}3\times10^6$ and $0{\cdot}7\times10^6$ daltons, respectively (Loening, 1968). The precursor rRNA components will be referred to by their molecular weights: sedimentation coefficients are used only when discussing earlier results obtained by centrifugation.

3. Results

(a) *Molecular weights of the precursor components*

A gel electrophoretic-separation of the rapidly-labelled RNA of artichoke tissue is shown in Figure 1. The radioactivity profile shows four discrete components with molecular weights of $2{\cdot}3\times10^6$, $1{\cdot}4\times10^6$, $0{\cdot}9\times10^6$ and $0{\cdot}7\times10^6$ daltons. The first three of these and much of the heterogeneous RNA, but not the labelled $0{\cdot}7\times10^6$ daltons rRNA, were recovered in the crude nuclear preparations (Fig. 1(b)). The cytoplasmic fraction contained most of the labelled $0{\cdot}7\times10^6$ daltons rRNA.

Rapidly-labelled RNA from the pea-root tip shows the same components as that from artichoke tissue. However, the $2{\cdot}3\times10^6$ daltons RNA peak is less stable. Extensive breakdown occurred in earlier experiments, so that there was a broad peak of radioactivity at about $2{\cdot}1\times10^6$ daltons, trailing to lower molecular weights. The use of the homogenization medium described above prevented such breakdown. Separation of the pea-root homogenate into nuclear debris and cytoplasmic supernatant fractions again shows that the $2{\cdot}3\times10^6$, $1{\cdot}4\times10^6$ and $0{\cdot}9\times10^6$ daltons RNA's are concentrated in the nuclear fraction, whereas labelled $0{\cdot}7\times10^6$ daltons rRNA rapidly enters the

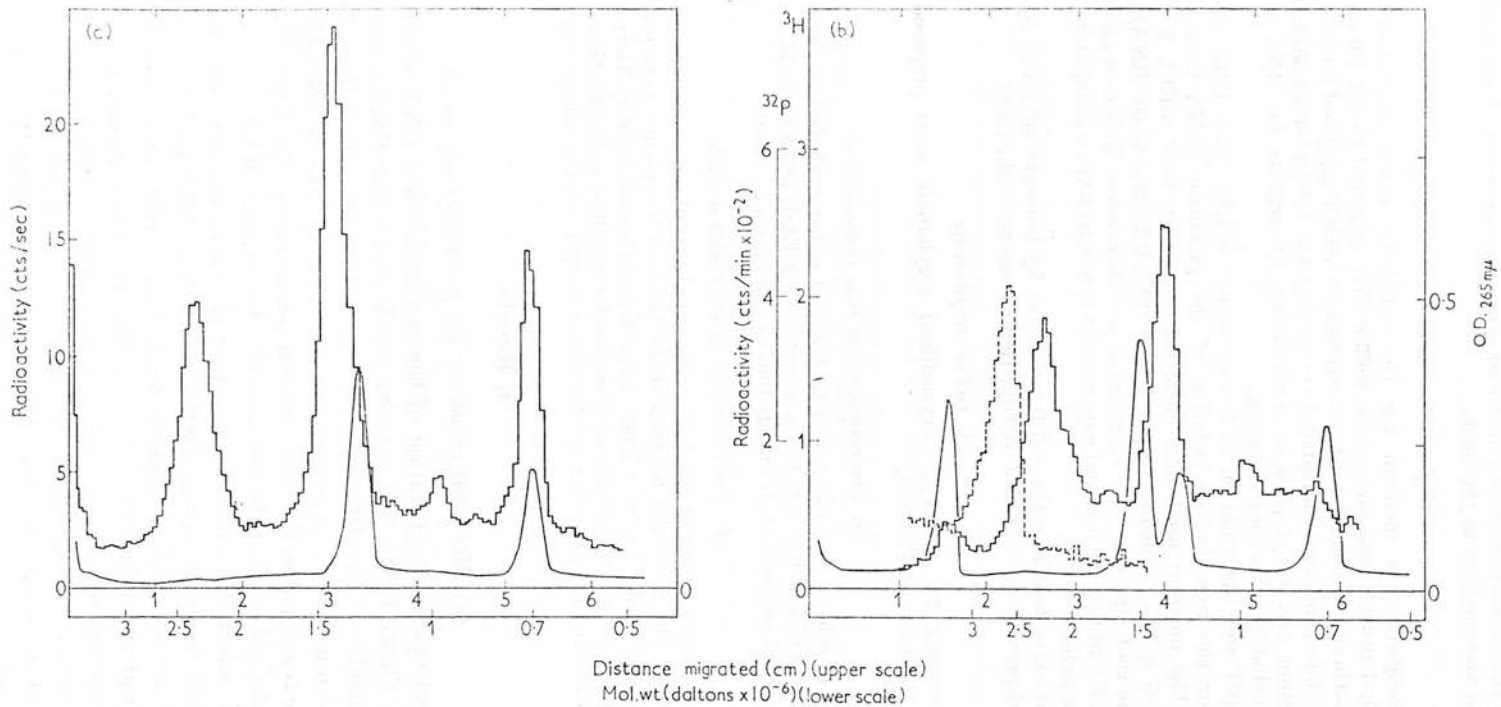

FIG. 1. Rapidly-labelled RNA in artichoke tuber tissue. Tuber explants were incubated in ^{32}P after 30 hr growth; this time follows 1 cell division. (a) 1-hr incubation (15 min in ^{32}P followed by 45-min chase in normal medium); total nucleic acids extracted from the tissue without cell fractionation and DNA removed with DNase. Electrophoresis in a 2·4% gel at 19 v for 12 hr. (b) 45-min incubation (25-min pulse followed by 20-min chase); RNA extracted from the nuclear fraction and DNA not removed. The sample was mixed with tritiated *Xenopus* RNA as a marker, prepared as previously described (Loening *et al.*, 1969). Electrophoresis was in a 2·2% gel at 50 v for 3 hr. (————) Optical density at 265 mμ; ___⌐¬___ histogram, cts/sec ^{32}P-labelled RNA; ___⌐¬___ cts/sec ^{3}H-labelled RNA (*Xenopus*).

cytoplasm. For this reason, and others to be discussed, we suggest that the $2{\cdot}3\times10^6$ daltons RNA is a precursor to both RNA's, that the $1{\cdot}4\times10^6$ daltons RNA is the immediate precursor to $1{\cdot}3\times10^6$ daltons rRNA, and that $0{\cdot}9\times10^6$ daltons RNA is the immediate precursor to $0{\cdot}7\times10^6$ daltons rRNA.

The $2{\cdot}3\times10^6$ daltons RNA component from the pea-root tip is broader than might be expected for a homogeneous RNA, both in preparations of total RNA from the whole tissue (Loening *et al.*, 1969) and in nuclear fraction RNA. The heterogeneity can be seen most clearly in the optical-density profiles of electropherograms of nuclear RNA as in Figure 2. At least two distinct components are apparent, which differ by

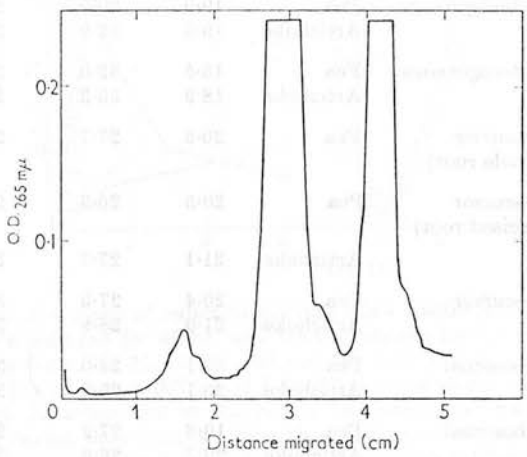

FIG. 2. Optical-density profile of scan of pea nuclear RNA.

Roots were cut at 0°C into segments as described. A nuclear fraction was prepared by homogenization and centrifugation; RNA was extracted and DNA digested. The Figure shows the optical-density scan of the nuclear RNA obtained from 56-sec root segments. Electrophoresis in 2·2% gel for 3·5 hr. (———) Optical density at 265 mμ.

$0{\cdot}1\times10^6$ daltons in molecular weight. This profile was also reproducible in preparations from different regions of the root.

In pea and artichoke tissue, extraction of the remaining protein interface with a further volume of Parish & Kirby (1966) detergents containing 0·5 M-NaCl, at 55°C for two minutes, yielded an additional amount of the $2{\cdot}3\times10^6$ daltons RNA. This was only 5 to 10% of the yield obtained with the cold detergents, which may therefore be assumed to extract the bulk of this precursor.

(b) *Base compositions*

The compositions of the precursor components show that they are similar to rRNA and clearly distinguishable from the heterogeneous RNA (Table 1). The guanine content of the $2{\cdot}3\times10^6$ daltons precursor is slightly less than that of the weighted average of the two ribosomal RNA's. This could partly be due to contamination by the underlying heterogeneous RNA. The data are not sufficiently precise to enable one to conclude the composition of the non-ribosomal excess RNA in the precursor.

The composition of the $1{\cdot}4\times10^6$ daltons component is similar to that of the $1{\cdot}3\times10^6$ daltons rRNA. This suggests that it is the immediate precursor to the rRNA, and not a dimer produced by aggregation of the $0{\cdot}7\times10^6$ daltons rRNA.

TABLE 1

Base composition of pea and artichoke RNA components

Mole wt of RNA (daltons $\times 10^6$)	Type of RNA		Moles per cent C	A	G	U	G + C
	Short label (45 to 60 min)						
>3	Heterogeneous	Pea	19·9	30·0	25·0	25·1	44·9
		Artichoke	19·5	32·0	26·9	21·8	46·4
<2	Heterogeneous	Pea	18·5	32·0	24·9	24·6	43·4
		Artichoke	18·9	33·3	26·8	21·2	45·7
2·3	Precursor (whole root)	Pea	20·5	27·7	28·8	22·9	49·3
2·3	Precursor (excised root)	Pea	20·5	26·3	29·4	23·8	49·9
		Artichoke	21·1	27·7	30·0	21·3	51·1
1·4	Precursor	Pea	20·4	27·5	31·5	20·6	51·9
		Artichoke	21·0	28·8	31·3	18·7	52·3
1·3	Ribosomal	Pea	20·1	28·0	31·8	20·1	51·9
		Artichoke	20·1	29·2	31·1	19·4	51·2
0·7	Ribosomal	Pea	19·8	27·9	28·3	23·3	48·1
		Artichoke	20·3	26·9	28·3	24·5	48·6
	Long label (>2 h)						
1·3	Ribosomal	Pea	21·8	24·9	32·2	21·1	54·0
		Artichoke	21·6	28·1	31·5	18·9	53·1
0·7	Ribosomal	Pea	21·1	25·9	28·5	24·4	49·6
		Artichoke	20·5	27·6	28·1	23·9	48·6
Weighted-average of rRNA's: (pea)			21·6	25·3	30·9	22·2	52·5

Gel slices were taken from regions indicated by molecular weight. 1 to 3 peak slices were used for the ribosomal and precursor components, and 2 to 5 for the heterogeneous components. Standard deviation were up to $\pm 0{\cdot}4$ for peak fractions, and $\pm 0{\cdot}8$ for heterogeneous RNA. The number of determinations was 3 to 6 in each case. A minimum of 2000 counts above background (5 cts/min) was collected per sample.

(c) *Time-course of labelling of rRNA*

In pulse–chase incubations of artichoke tissue, it was shown that the labelling of rRNA continues for several hours after a 15-minute pulse while that of DNA ceases after 30 minutes. The incorporation of ^{32}P into the rRNA and precursors in the nuclear and cytoplasmic fractions is shown in Figure 3(a) and (b). The $2{\cdot}3 \times 10^6$ daltons precursor became labelled rapidly during the first 40 minutes of incubation, and lost label after longer chase incubations. This is consistent with its precursor function, although further data would be required to demonstrate a direct precursor relationship to the rRNA. Artichoke tissue differs from many other plant tissues in that it is possible to demonstrate the loss of label from the precursor during the chase incubation.

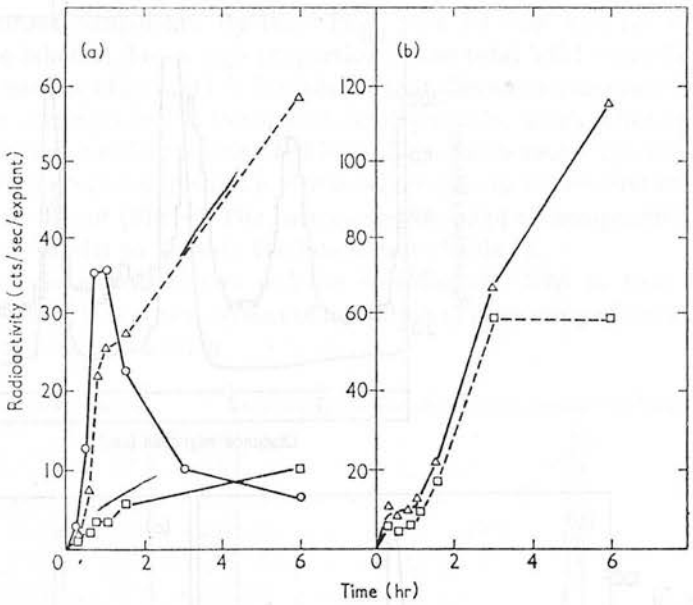

FIG. 3. Uptake of radioactivity into RNA fractions from the artichoke.
Explants were cultured for 40 hr, and then incubated for 15 min in ^{32}P. Subsequent chase incubations were for 1, 15, 30, 45, 75, 165 and 345 min. The cytoplasmic fraction was variably contaminated with precursor RNA and $1{\cdot}3\times10^6$ daltons rRNA owing to the method of homogenization used. Radioactivity in the rRNA and precursors was obtained from the electrophoresis scans, taking only counts above the heterogeneous background. (a) Nuclear fraction RNA; (b) cytoplasmic fraction RNA. (a) —○——○—, $2{\cdot}3\times10^6$ daltons; —△——△—, $1{\cdot}3\times10^6$ daltons (plus $1{\cdot}4\times10^6$ daltons); —□——□—, $0{\cdot}7\times10^6$ daltons. (b) —△——△—, $1{\cdot}3\times10^6$ daltons; --□--□--, $0{\cdot}7\times10^6$ daltons.

Label in the $1{\cdot}4\times10^6$ and $1{\cdot}3\times10^6$ daltons components (these were summed in Figure 3(a), since they have not been completely separated by gel electrophoresis) increased for several hours during the chase incubation. The $1{\cdot}4\times10^6$ daltons component was labelled before the $1{\cdot}3\times10^6$ daltons rRNA, as indicated for a 45-minute incubation in Figure 1. Nuclear $0{\cdot}7\times10^6$ daltons rRNA is labelled very slowly, as indicated in Figure 1 (the pea and artichoke are similar) and Figure 3. It is probable that the specific activity of the $0{\cdot}7\times10^6$ daltons rRNA is lower in the nuclear fraction than in the cytoplasmic for incubation times up to about two hours.

(d) *Ribosomal RNA synthesis in different regions of the pea root*

Rapidly-labelled RNA of the three root segments was investigated in preparations of total RNA without cell fractionation, thus minimizing degradation. An approximate measure of the rate of processing of the $2{\cdot}3\times10^6$ daltons precursor was obtained from the ratio of the label in rRNA (here including $1{\cdot}4\times10^6$ daltons RNA) to that in the precursor. With short incubations of up to 40 minutes, during which the label in all components is increasing, a higher ratio would indicate faster processing or a shorter lifetime of the primary precursor. Figure 4 shows that after a 35-minute incubation, the ratio of label in $1{\cdot}4\times10^6$ and $0{\cdot}7\times10^6$ daltons rRNA to that in $2{\cdot}3\times10^6$ daltons RNA is similar in all three segments. After a 15-minute incubation only the $2{\cdot}3\times10^6$ daltons precursor was labelled in all three segments. Assuming that the ratios of label

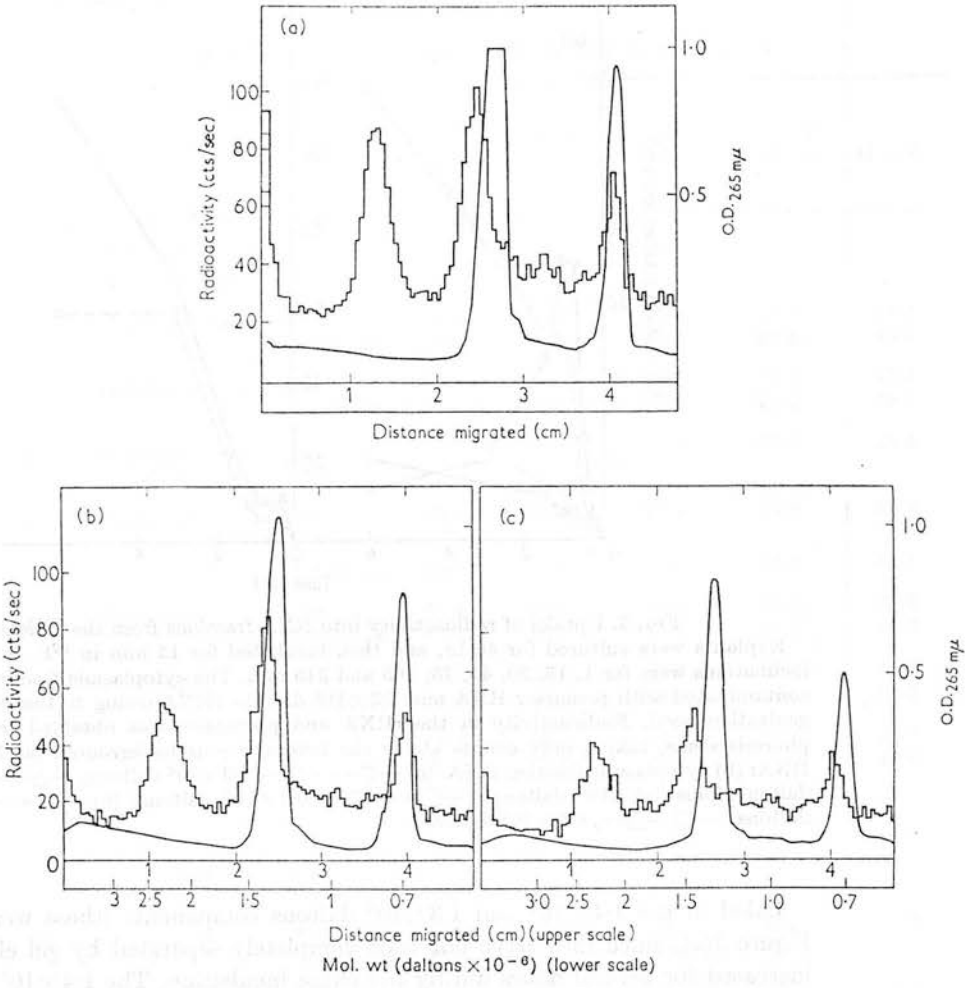

FIG. 4. Timing of rRNA synthesis in different regions of the pea root.
Seedlings were labelled in ^{32}P (0·6 mc) as described in the text, for 15 min, and incubation was continued in 10^{-5} M-phosphate for 20 min. Each root was then cut at 0°C into 3 segments as described in the text; (a) the tip, 0 to 1·6 mm, (b) 1·6 to 3·4 mm, (c) 3·4 to 6·4 mm, using a Plexiglass template. Total RNA was extracted and DNA digested. RNA equivalent to the yield from about six segments was applied to each gel. Electrophoresis was in 2·4% polyacrylamide gels for 3 hr. The logarithmic molecular-weight scale is indicated except where the exact position of optical-density peaks cannot be determined. (———) Optical density at 265 mμ; (⎍⎍) histogram, cts/sec ^{32}P.

are independent of any differences in uptake of ^{32}P into the three segments, the results indicate that the rate of processing is as high in the older parts of the root as in the younger.

(e) *Ribosomal RNA synthesis in excised pea-root tips*

Root tips were incubated in ^{32}P in sucrose for one hour immediately after excision, followed by various times in sucrose alone. After 30 minutes in ^{32}P, only the $2{\cdot}3 \times 10^6$ daltons precursor and heterogeneous RNA were labelled, as in the intact root after 15 minutes incubation. After one hour, label was just detectable above background

in the $1{\cdot}4\times10^6$ daltons component. By three hours, the $1{\cdot}4\times10^6$ and the $0{\cdot}7\times10^6$ daltons rRNA were labelled, but a high proportion of the total label was still in the $2{\cdot}3\times10^6$ daltons precursor (Fig. 5(a)). It is probable that this has accumulated by this time, since it can be distinguished in the optical-density profile, which is not normally possible in preparations of unfractionated cell RNA from whole roots. The amount of label in the $0{\cdot}9\times10^6$ daltons component is also much greater in the excised root (Fig. 5(a)) than in the intact root (Fig. 4). The base compositions of all components in the excised root tip were similar to those in the intact root (Table 1).

A comparison between these results and the labelling of rRNA in root tips of intact seedlings (Fig. 5(b)) shows the extent of inhibition of processing of the $2{\cdot}3\times10^6$ daltons precursor caused by excision.

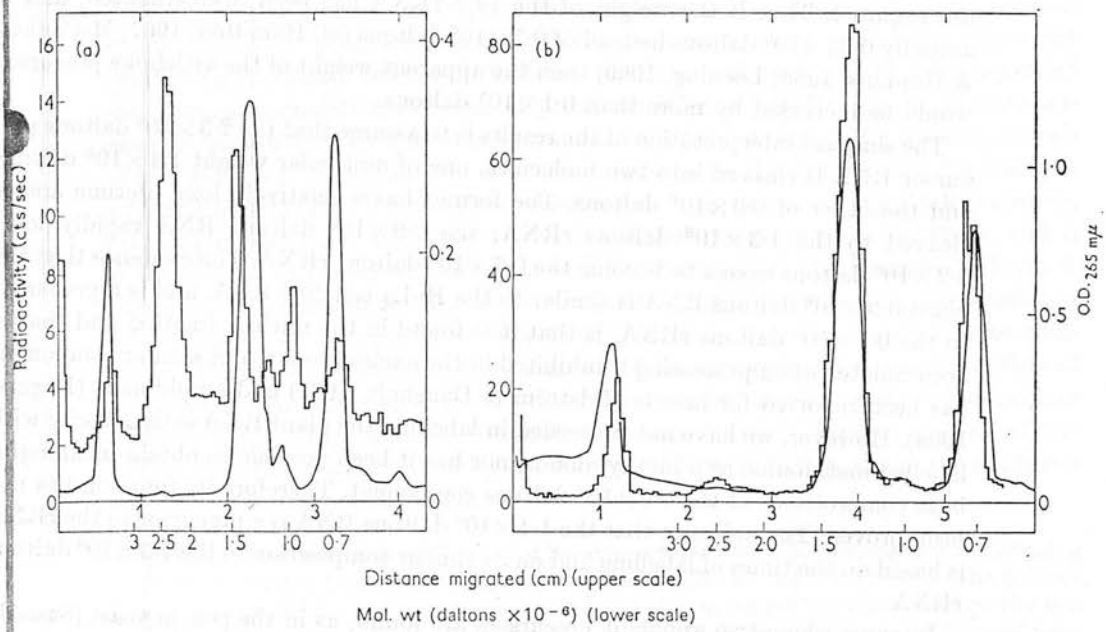

FIG. 5. Timing of rRNA synthesis in the cultured pea root tip.

(a) Root tips measuring 3 mm were labelled for 1 hr in small Petri dishes, containing 20 tips/ml. 2% sucrose/dish, and 0·75 mc ^{32}P/dish. Incubation was continued in 2% sucrose for 2 hr. RNA was extracted as described, but DNA was not digested. RNA equivalent to the yield from 2 roots was applied to the gel. Electrophoresis was for 3 hr in 2·2% gels.

(b) Labelling of rRNA over long periods in the intact seedling. Seedlings were labelled in 2 mc ^{32}P for 45 min, followed by further incubation in water for 3 hr. Root tips measuring 3 mm were cut off, and their RNA extracted. Electrophoresis was for 3·5 hr in 2·2% gels. (———) Optical density at 265 mμ; (⎍⎍) histogram, cts/sec ^{32}P-labelled RNA.

4. Discussion

The results show that a high molecular weight precursor to rRNA can be found in plant tissues. It appears to be heterogeneous in some species and its molecular weight varies from $2{\cdot}3\times10^6$ to $2{\cdot}8\times10^6$ daltons in different species. The weight of the precursor in mung bean leaves is about $0{\cdot}3\times10^6$ daltons more than that in the artichoke (Loening *et al.*, 1969). In the carrot (Leaver & Key, 1970) and in yeast (Sassella & Loening, manuscript in preparation) there seem to be two distinct components. Their

molecular weights are $0{\cdot}3\times10^6$ to $0{\cdot}8\times10^6$ daltons larger than the sum of the weights of the rRNA components, and the excess is presumably lost during processing. This amount of excess RNA is much smaller than that in mammals and compares to that in *Xenopus* (Loening *et al.*, 1969). In all these eukaryotes, it appears to be a universal phenomenon that the smaller rRNA, $0{\cdot}7\times10^6$ daltons, is rapidly transported to the cytoplasm, while the immediate precursor to the larger rRNA remains for an hour or more in the nucleolus.

An exact interpretation of these results in terms of the probable stages of processing depends critically on the accuracy of the molecular-weight determinations. The gel-electrophoretic method essentially determines ratios of molecular weight between components, while it is differences of weight between precursors and products which are required. Thus if the weight of the 18 s rRNA has been overestimated, and is actually $0{\cdot}65\times10^6$ daltons instead of $0{\cdot}7\times10^6$ daltons (cf. Hamilton, 1967; McConkey & Hopkins, 1969; Loening, 1969) then the apparent weight of the artichoke precursor would be increased by more than $0{\cdot}1\times10^6$ daltons.

The simplest interpretation of the results is to assume that the $2{\cdot}3\times10^6$ daltons precursor RNA is cleaved into two molecules, one of molecular weight $1{\cdot}4\times10^6$ daltons and the other of $0{\cdot}9\times10^6$ daltons. The former has a relatively long lifetime and is cleaved to the $1{\cdot}3\times10^6$ daltons rRNA; the $0{\cdot}9\times10^6$ daltons RNA rapidly loses $0{\cdot}2\times10^6$ daltons excess to become the $0{\cdot}7\times10^6$ daltons rRNA. The evidence that the plant $0{\cdot}9\times10^6$ daltons RNA is similar to the HeLa cell 20 s RNA, and is a precursor to the $0{\cdot}7\times10^6$ daltons rRNA, is that it is found in the nuclear fraction and that it accumulates when processing is inhibited in the excised root tip. A similar component has been reported for insects (Edström & Daneholt, 1967) and amphibians (Rogers, 1968). However, we have not succeeded in labelling the plant RNA satisfactorily with labelled methionine as a methyl donor, nor has it been possible to obtain meaningful base compositions of the $0{\cdot}9\times10^6$ daltons component. Therefore its function has not been proved. The evidence that the $1{\cdot}4\times10^6$ daltons RNA is a precursor to the rRNA is based on the times of labelling and on its similar composition to the $1{\cdot}3\times10^6$ daltons rRNA.

In cases where two apparent precursors are found, as in the pea, in yeast (Sassella & Loening, manuscript in preparation) and in carrot (Leaver & Key, 1970), it would be the one of lower molecular weight which would be split exactly into the two immediate precursors to the $1{\cdot}3\times10^6$ and $0{\cdot}7\times10^6$ daltons rRNA's. The higher molecular-weight precursor would then include a variable amount of excess RNA above the minimum weight of $2{\cdot}3\times10^6$ daltons. This would account for the heterogeneity of the precursor as well as for the differences between species.

The heterogeneity could be due to the synthesis of molecules of different length, to a gradual rather than stepwise loss of the excess RNA, or to processing before synthesis is completed. These alternatives cannot be distinguished at present. However, in pea and carrot the two precursors can be detected by their optical densities on the gels, and should therefore have comparable lifetimes. The pulse incubations (Leaver & Key, 1970) do not distinguish between them, and so suggest that at least some of the lower molecular-weight molecules are synthesized directly, independently of larger molecules.

If the above interpretation is correct, then the $2{\cdot}3\times10^6$ daltons precursor can be compared to the 41 s ($3{\cdot}1\times10^6$ to $3{\cdot}4\times10^6$ daltons) component in HeLa cells, which is cleaved to 32 s ($2{\cdot}1\times10^6$ to $2{\cdot}4\times10^6$ daltons) and 20 s ($0{\cdot}9\times10^6$ to 1×10^6 daltons)

without loss of excess RNA (Weinberg & Penman, 1970). All the eukaryotes would then have in common a ribosomal precursor of a minimum molecular weight sufficient to give the immediate precursors to the rRNA. This minimum is $2{\cdot}3 \times 10^6$ daltons in plants, $2{\cdot}5 \times 10^6$ daltons in *Xenopus* (Loening *et al.*, 1969) and about $3{\cdot}3 \times 10^6$ daltons in HeLa cells (Weinberg & Penman, 1970). Different species have a first precursor which contains a variable excess of RNA above this minimum, from 0 to $0{\cdot}5 \times 10^6$ daltons in plants, about $0{\cdot}1 \times 10^6$ daltons in *Xenopus* to about 1×10^6 daltons in mammals. In birds, the excess appears to be slightly smaller than in mammals (Perry *et al.*, 1970).

These results support the suggestion made previously (Loening *et al.*, 1969; Perry *et al.*, 1970) that the evolution of the mammalian precursor is the result of a shift of the initiation or termination site to previously non-transcribed lengths of the ribosomal DNA.

The growth of the pea root provides opportunities for the study of the rates of synthesis and of processing of the rRNA precursors under conditions where net synthesis is decreased. Our results indicate that in the older cells, which have expanded and become vacuolated, the pool of precursor RNA is smaller and its lifetime unchanged or shorter. The control of synthesis must then be close to the site of synthesis of the precursor. In contrast to this, the conditions in the excised root tip result in a pool of precursor with greatly increased life, or delay in processing. In this system, net protein and RNA synthesis are inhibited, but cell expansion continues, and is in some cases increased by inhibitors of RNA metabolism like thiouracil (Heyes & Vaughan, 1967) and ribonuclease (Yeoman, 1962). Since enzyme turnover continues, and radioactivity is incorporated into DNA-like RNA, the excised root may be compared to a step-down system (Loening, 1965). It is possible that the inhibition of processing is through a shortage of ribosomal proteins, in which case the immediate effect of excision suggests that the pool of proteins is small. Further analysis of this and other plant systems should yield considerable information about the synthesis and transport of ribosomal RNA.

We thank Professor Robert Brown for his support and Dr M. M. Yeoman for introducing us to the artichoke tissue culture system. One of us (M. E. R.) is grateful to the Science Research Council for a NATO post-doctoral fellowship in support of work done on the pea root. One of the other authors (R. S. S. F.) is grateful to the Science Research Council for a studentship in support of work on the artichoke; this work was carried out in partial fulfilment of the requirements for the Ph.D. degree, Edinburgh University, Scotland.

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Extract from
Journal of Experimental Botany
Vol. 25, No. 88

Oxford : at the Clarendon Press

Journal of Experimental Botany, Vol. 25, No. 88, pp. 847–59, October 1974

RNA Synthesis during Synchronous Cell Division in Cultured Explants of Jerusalem Artichoke Tuber

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Received 12 March 1974

ABSTRACT

Explants of Jerusalem artichoke tuber tissue were cultured in nutrient medium with the hormone, 2,4-dichlorophenoxyacetic acid. After a lag period, 90 per cent of the cells divided synchronously. During the first two cell cycles, the rate of ribosomal RNA synthesis increased sharply in two steps; before the onset of DNA synthesis for the first division, and early in interphase before the second division. Rates of RNA and protein accumulation, and phosphate uptake also increased sharply at these times. From experiments with explants in which DNA synthesis and cell division had been inhibited, it was concluded that the stepwise pattern of ribosomal RNA synthesis was not caused by the replication of ribosomal RNA genes, as can happen in mammalian cells. Instead, the periodicity of metabolism was found to be independent of the DNA synthesis-cell division cycle. A cause of the stepwise nature of ribosomal RNA synthesis is suggested.

It is considered that despite the high synchrony of division, the system is not completely suited for the study of events associated with the cell cycle in higher plants. However, the synchrony of much of early metabolism suits it to the study of induction of cell division in previously non-dividing cells, and the consequent process of de-differentiation.

INTRODUCTION

The normal fate of the storage tissue of Jerusalem artichoke tuber is to break down to provide nutrients for the developing buds. But when explants of tuber tissue are placed in a suitable nutrient medium, vigorous cell division is induced (Yeoman, Dyer, and Robertson, 1965). All but 1 per cent of the cells of the freshly-cut explant are highly vacuolated, storage parenchyma. Repeated division, with little accompanying cell expansion, leads to a tissue of small, non-vacuolated, meristematic-type cells (Yeoman *et al.*, 1965). The growth of explants thus displays two features characteristic of oncogenesis, an increased rate of cell division and de-differentiation.

The first few divisions in the explant are inherently synchronous (Yeoman and Evans, 1967, and Fig. 1). DNA synthesis for these divisions is also synchronous (Mitchell, 1967). Under the correct culture conditions, 80–90 per cent of the cells divide (Fraser, Loening, and Yeoman, 1967). The synchrony of division, and the high uniformity of cellular composition and response suggest that the system provides a good opportunity to study metabolic events connected with the cell

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cycle, and with the transition of cells from a differentiated state to a de-differentiated one. It is of interest to ask whether changes in aspects of metabolism other than cell division also occur in a synchronous manner, and to determine whether these changes are related to the cell division cycle or to some overall growth pattern of the explant.

We have studied the rate of ribosomal RNA (r-RNA) synthesis during the first two divisions, and have followed changes in explant contents of r-RNA, transfer RNA (t-RNA), and protein. We then tested the extent to which these changes were related to cell division and the cell cycle rhythm by experiments with explants in which DNA synthesis and cell division had been inhibited.

MATERIALS AND METHODS

Tissues

Explants of secondary xylem parenchyma were removed aseptically, in dim green light (Fraser *et al.*, 1967) from tubers of Jerusalem artichoke (*Helianthus tuberosus* L. var. 'Bunyard's Round'). The explants were 9 mg fresh weight and contained about 20 000 cells. The completely defined culture medium contained mineral salts (Bonner and Addicott, 1937) 4% sucrose, and 10^{-5} M 2,4-dichlorophenoxyacetic acid (2,4-D). Thirty explants were cultured in 5·25 ml sterile medium in a 90 mm diameter petri dish, in total darkness. The cultures were agitated on a reciprocating shaker operating with a 50 mm displacement at 50 cycles/min.

Radioactive incubation

Batches of 30 explants were rinsed with phosphate-free culture medium to remove intercellular phosphate. The 'pulse' incubation was for 15 min in 3 ml culture medium lacking phosphate, but containing 85 μCi/ml ^{32}P-orthophosphate (Radiochemical Centre, Amersham, U.K.). A 75 minute 'chase' incubation followed in 10 ml non-radioactive, complete culture medium, which had a phosphate concentration equivalent to 12 mgl^{-1} KH_2PO_4. All solutions used in labelling were sterile.

The 'chase' incubation in this tissue is highly effective in preventing further entry of ^{32}P into nucleotide precursor pools. After a 90 min pulse-chase incubation, about 75 per cent of the total counts in r-RNA were in the mature 25 and 18S species, while 25 per cent were in the r-RNA precursor. Full details of the kinetics of labelling are published in Rogers, Loening, and Fraser, 1970.

Extraction and fractionation of nucleic acids

Nucleic acids were extracted by a detergents-phenol procedure (Kirby, 1965; Loening, 1967) and precipitated with ethanol. The extraction method used gave a high, reproducible yield of undegraded RNA (Fraser, 1968, 1971). An estimate of ^{32}P uptake by the explants was obtained by counting samples of the supernatant remaining after sedimentation of the precipitated nucleic acids. The nucleic acids were further purified by successive reprecipitations (Loening, 1969).

Nucleic acids were fractionated by electrophoresis on polyacrylamide gels (2·2% acrylamide, 0·11% bis-acrylamide) for 90 min at 5 mA/gel, 8 V/cm gel length (Loening, 1967). The gels were scanned for ultraviolet absorption at 265 nm with a Joyce-Loebl Chromoscan. The gels were then frozen in solid CO_2 and sliced transversely at 0·5 mm intervals with a Mickle gel slicer. Radioactivity in each slice was determined in a Beckman Lowbeta planchet counter. Fig. 2 shows a typical gel of nucleic acids from explants labelled during the first period of DNA synthesis.

The following data were derived from such gel scans. The weights of r-RNA (18S plus 25S) and t-RNA were calculated from the areas of their peaks on the absorbance scan. Peak area is linearly proportional to the weight of RNA present (Ingle, 1968; Fraser, 1971). By this means it was possible to calculate explant contents of r-RNA and t-RNA. The total radioactivity incorporated into r-RNA was found by adding the radioactivities of the individual gel slices

in the 25S and 18S radioactivity peaks, allowing for the level of polydisperse background labelling on the gel (Fraser, 1973).

Estimation of total DNA, RNA, and protein contents

Explants were stored in methanol at 0 °C between harvesting and analysis. Ten explants were used for each determination. Washing to remove contaminants, such as lipids and nucleotides, was by the method of Holdgate and Goodwin (1965). The explants were not homogenized for washing or subsequent extraction of nucleic acids. Homogenization did not increase the efficiency of washing or extraction, and gave less reproducible results.

Nucleic acids were extracted into 0·5 N $HClO_4$ by five successive 20 min extractions at 70° C (Schneider, 1945). RNA concentration was calculated from the ultraviolet absorption spectrum of the combined first and second extracts. The 260:235 nm absorbance ratios of the extracts were over 2:1. DNA was determined on the combined five extracts by the diphenylamine method (Burton, 1956). Although $HClO_4$ extracts contain both RNA and DNA, RNA is about 90 per cent of the total nucleic acid present in explants, and RNA is extracted much more rapidly than DNA by $HClO_4$ (Fraser, 1968). The first two extracts therefore contained only a few per cent of DNA, and thus provided a suitable measure of total RNA.

Protein was extracted from the remains of the explants by incubating with 1 ml N NaOH for 12 h at 20 °C, and was measured by the method of Lowry, Rosebrough, Farr, and Randall (1951).

Each total RNA, DNA, or protein value quoted in the results is the mean of at least three replicate determinations. Over 90 per cent of the individual determinations were within ±5 per cent of the mean.

Cell number and the synchrony of cell division

Cell numbers were estimated on samples of five explants, macerated for 24 h at 20 °C in 2 ml 5% chromic acid (Brown and Rickless, 1949). Chromic acid does not degrade the wall of the original cells of the explant (Yeoman and Evans, 1967). Thus when a cell has divided once during culture, the products appear in the macerate as a 'pair', that is as two distinct daughter cells within an outer cell wall. Similarly, the second division leads to the appearance of 'fours', the third division to 'eights' etc. This enabled us to quantify progress of the first and second divisions, and thus to confirm the high degree of synchrony of division.

By scoring a sample of macerate for total cells (T); single, undivided cells, and the numbers of 'pairs' (P) and 'fours' (F) etc., it was possible to calculate the increase in cell number which had occurred during culture in the sample examined: $I = 1 \times P + 3 \times F$ etc. The number of cells in that sample at the start of culture is $X = T - I$.

From these values, and expressing the results per 100 cells present at zero time, we can derive data for the total number of cells in the explant: $(T \times 100)/X$; the percentage increase in cell number during culture: $(I \times 100)/X$; the number of cells arising as a consequence of the first division: $(2P \times 100)/X$; and the number of cells arising as a consequence of the second division: $(4F \times 100)/X$.

Figure 1 shows an analysis of changes during culture in the cellular composition of explants. After a lag of 22 h during which no division occurred, 85 per cent of the cells completed the first division between 22 and 28 h. In other words, up to 22 h, no 'pairs', indicative of once-divided cells, were visible in the macerates; between 22 and 28 h 85 per cent of the original cells became 'pairs' in the macerate. The 15 per cent of cells which did not enter the first division did not divide later. Between 28 and 35 h, there was no cell division in the explants. The number of cells as 'pairs' remained constant, and no 'fours' appeared. At 35 h, the second division commenced: 'fours' began to appear in the macerates and increased to 44 h. The number of cells as 'pairs' correspondingly fell as 'fours' appeared, but some cells which had completed the first division failed to divide again. This is shown by the persistence of a residual population of 'pairs' after the second division. 'Eights', indicating the occurrence of a third division, began to appear immediately after the end of the increase in 'fours'.

These data show that early division in the explant was highly synchronous, but that synchrony was lost as growth continued. The ability to recognize unequivocally the products of the first and second division made it possible to determine the timings of these divisions accurately, without having to perform the frequent cell counts necessary to demonstrate a step in cell number increase.

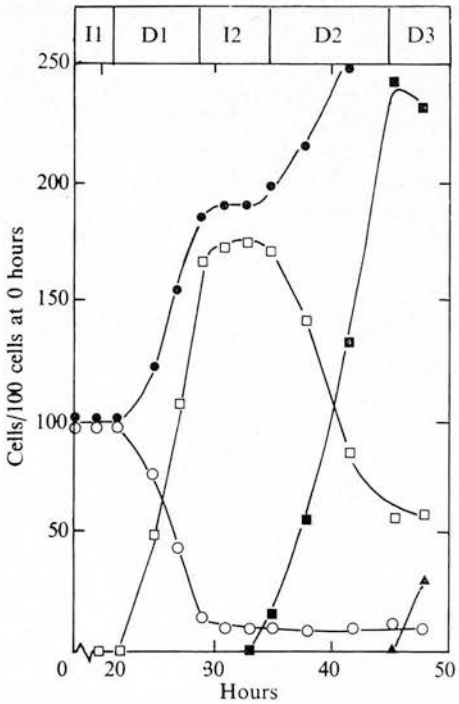

FIG. 1. Changes in cell number and cellular composition of explants with time of culture. ●—● Total cells; ○—○ single, (undivided) cells; □—□ cells present in the macerate as 'pairs', i.e. products of the first division; ■—■ cells present as 'fours', i.e. second division products; ▲—▲ cells present as 'eights', i.e. third division products. Counts were on samples of chromic acid macerate equivalent to 400 cells at 0 h; data were derived as explained in Materials and Methods. D1, D2, and D3 give the timings of the 1st, 2nd, and 3rd (cell) divisions; I1 and I2 are the interphases preceeding the 1st and 2nd divisions.

The accuracy of the method depends on there being no separation of daughter cells during maceration. Values of percentage increase in cell number derived by the method described agreed exactly with those calculated by estimating total cell numbers in explants before and after culture, by counting samples of macerate in a haemocytometer slide (Brown and Rickless, 1949). This confirms that no separation of daughter cells occurred during maceration, and that our method produced realistic cell number data.

Counting a sample of macerate equivalent to 400 original cells was found to produce highly reproducible results. In a control experiment involving counts of 10 samples of the same macerate, the percentage increase in cell number was 149 ± 3 (mean $\pm$ standard error). This level of reproducibility is higher than can easily be obtained by haemocytometer counting in this tissue (Evans, 1967).

The timing of division obtained by this method is the time of completion of cytokinesis. In this tissue, the mid-point of mitosis is 3 h before the completion of cytokinesis (Yeoman and Evans, 1967).

RESULTS

Timing of DNA synthesis and cell division

The period between excision and commencement of the first division was about 20 h in explants cut from newly harvested tubers, or from tubers stored for less than 6 months (Figs 1, 6). The pre-division lag period increased in length when the tubers had been stored for more than 6 months (e.g. Fig. 3). Once division had commenced, it was at the same rate irrespective of the history of the tubers (Evans, 1967).

Yeoman and Evans (1967) and Mitchell (1967) showed that DNA synthesis (S) for the first division takes 12–14 h. The period between the end of S and the first division (G_2) lasts for 0–2 h. Variation in the duration of the whole pre-division period is principally by variation in the length of the period (G_1) between excision and the commencement of DNA synthesis. DNA synthesis for the second division commences soon after the end of the first division, and occupies most of the interphase preceding the second division. We have used these results as the principal means of locating the times of DNA synthesis for the first and second divisions.

For confirmation of these timings, DNA synthesis was also estimated directly. DNA was detectable both as ultraviolet absorbance and radioactivity peaks on polyacrylamide gel fractionations of total nucleic acid from ^{32}P-labelled explants (Fig. 2). Unfortunately, it was not possible to derive satisfactory values for DNA amount or radioactivity from such gel separations. The method used to extract

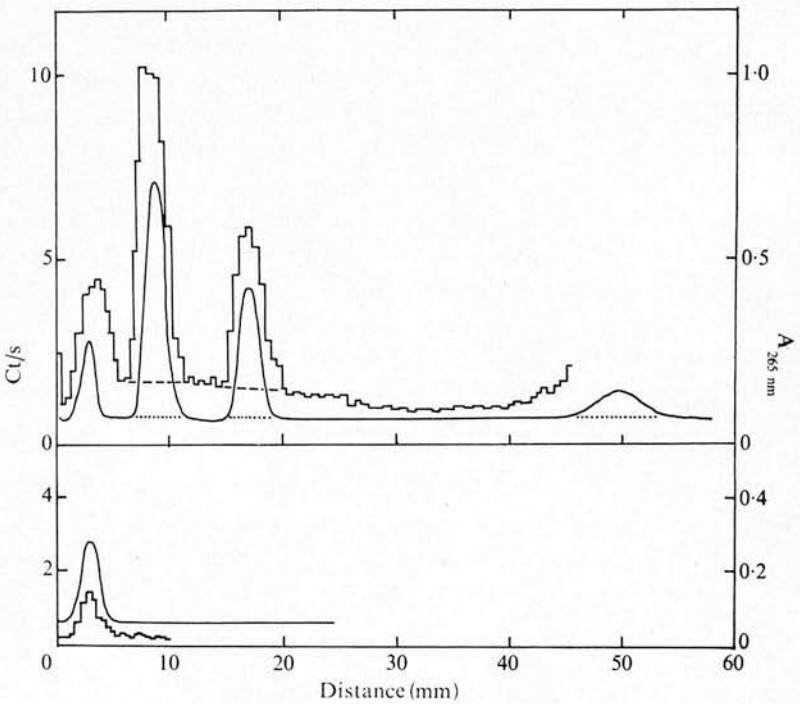

FIG. 2. Polyacrylamide gel electrophoresis of ^{32}P-labelled nucleic acids from explants labelled with ^{32}P during the first S period. The continuous lines show ultraviolet absorbance, the histograms show radioactivity. The peaks are: at 4 mm, DNA; at 9 and 17 mm, 26 and 18S r-RNA; at 50 mm t-RNA. The radioactivity peak at 5 mm includes the DNA and r-RNA precursor. The top panel shows total nucleic acid. The lower panel shows DNA, after removal of RNA by 10 μgml^{-1} pancreatic RNase at 20 °C for 20 min. The dotted lines show delimitation of ultraviolet absorption peaks for measurement of the weight of RNA on the gel; the broken lines show the delimitation of radioactivity peaks for estimation of the incorporation of ^{32}P into r-RNA.

RNA gave rather variable yields of DNA, and the radioactive r-RNA precursor (Rogers, Loening, and Fraser, 1970) on the gels obscured the DNA labelling. However, by electrophoresis of a sample of nucleic acid treated with RNase, the

radioactivity of the DNA could be determined (Fig. 2). This was expressed as a specific activity by dividing by the weight of DNA on the gel (calculated from the area of the DNA ultraviolet absorption peak). Thus errors arising from variation in DNA extraction were cancelled. This procedure was followed where it was important to confirm a timing, such as the beginning of DNA synthesis for the first division (cf. Fig. 3).

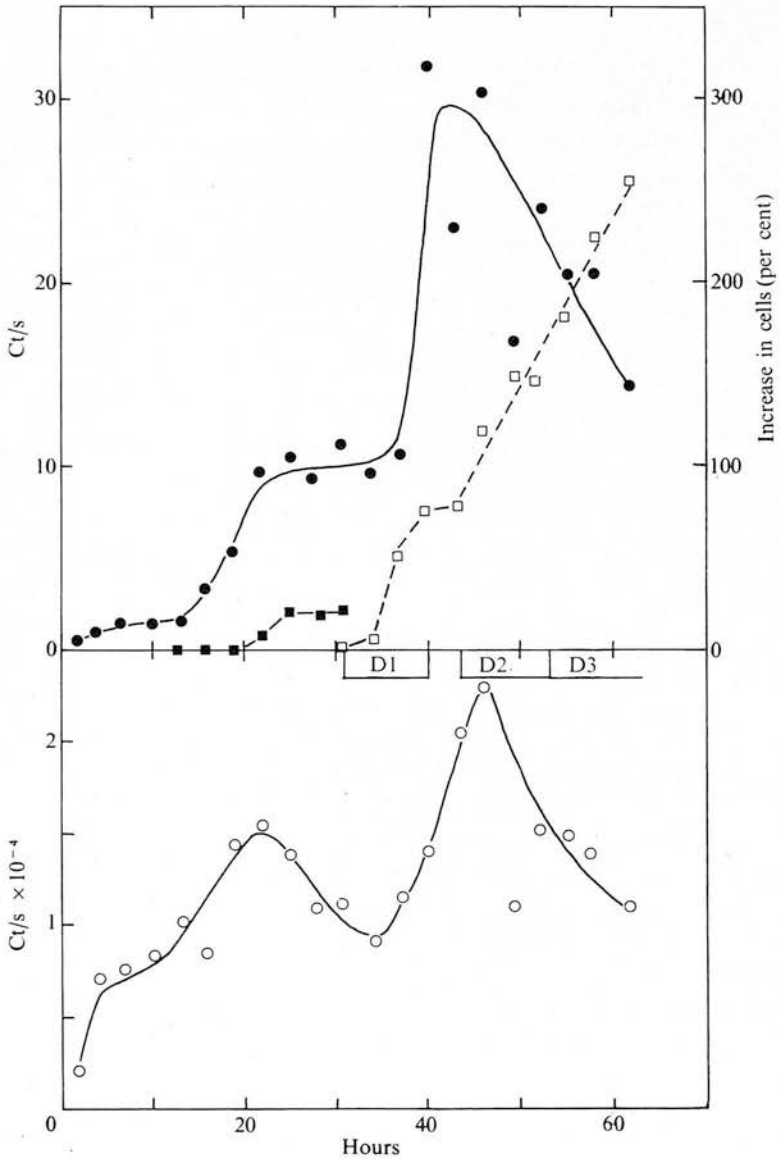

FIG. 3. Changes in the rates of r-RNA synthesis, DNA synthesis, and ^{32}P uptake during culture. ●—● Incorporation of ^{32}P into r-RNA (ct/s/explant); ■—■ incorporation of ^{32}P into DNA (ct/s/μg DNA); ○—○ ^{32}P uptake. □- -□ per cent increase in cell number. D1, D2, and D3 show the times of the first, second, and third divisions, identified as the times when 'pairs', 'fours', and 'eights' respectively were increasing in the chromic acid macerates.

The rate of r-RNA synthesis

The rate of r-RNA synthesis was measured after various times of culture, by incubating explants with ^{32}P for a total of 90 min, then determining the incorporation of ^{32}P into r-RNA.

In the explants used, the lag period before the first division was 34 h. The first division lasted from 34–39 h, and the second division had commenced by 45h (Fig. 3). From the results of Yeoman and Evans (1967) and Mitchell (1967), we calculated that DNA synthesis for the first division occurred between 20 and 35 h. The incorporation of ^{32}P into DNA, detected on polyacrylamide gels of RNase-treated total nucleic acid, commenced just after 20 h (Fig. 3).

r-RNA synthesis was very low for the first 14 h after excision (Fig. 3). Between 14 and 20 h, i.e. before the onset of DNA synthesis, the rate of r-RNA synthesis increased five-fold. During the first S phase, the rate of r-RNA synthesis remained constant. At the end of the first division, the rate increased again. Thereafter, the rate of r-RNA synthesis declined. Explants from older tubers, as these were, have only a limited capacity for growth and RNA accumulation when grown on completely defined medium.

Uptake of ^{32}P by the explants showed two rate maxima, at the times of the first and second rises in the rate of incorporation of ^{32}P into r-RNA (Fig. 3). The rate of uptake of ^{32}P declined during the first S period. The basic similarity in shape of the curves for ^{32}P uptake and ^{32}P incorporation into r-RNA suggested the possibility that changes in the rate of incorporation might reflect changes in uptake rather than changes in the rate of r-RNA synthesis. But when the incorporation data are replotted on a per unit ^{32}P uptake basis (Fig. 4), the changes during culture were similar to those of simple incorporation (Fig. 3), with two periods during culture when the rate increased. This suggests that the incorporation of ^{32}P into r-RNA is a meaningful measure of the rate of r-RNA synthesis.

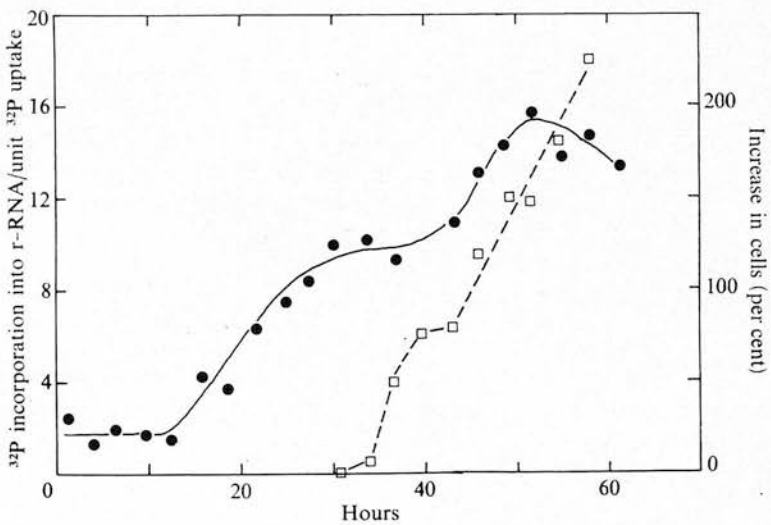

FIG. 4. Changes in the rate of r-RNA synthesis, expressed as ^{32}P incorporation into r-RNA per unit ^{32}P uptake, during culture (●—●). □--□ Percentage increase in cell number. The data were calculated from those of Fig. 3.

Accumulation of r-RNA, t-RNA, and protein

The r-RNA content of explants remained steady for the first 20 h, then increased sharply by about 50 per cent (Fig. 5). This increase occurred shortly after the first increase in the rate of r-RNA synthesis, which was measured on the same cultures

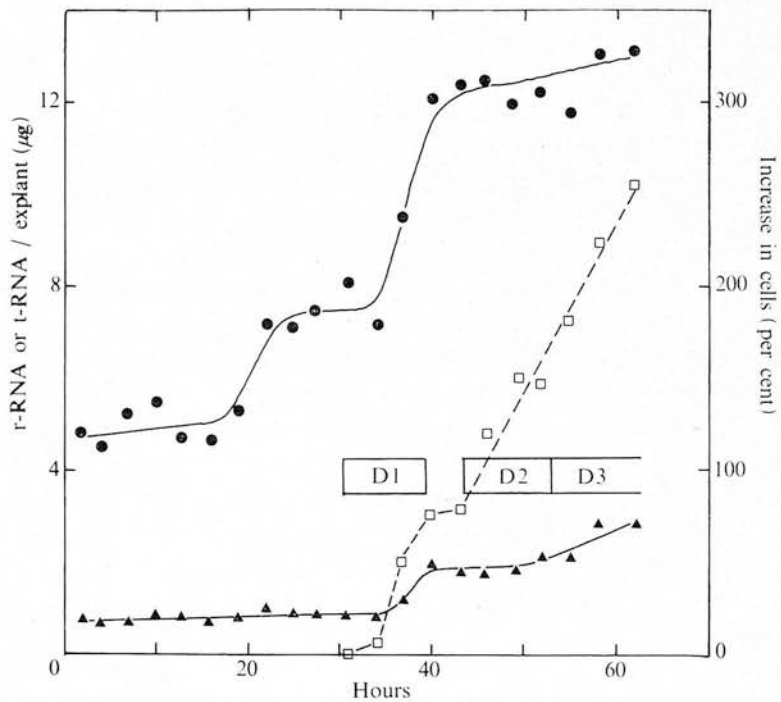

FIG. 5. Changes in the weight of r-RNA (●—●) and t-RNA (▲—▲) per explant with time. □- -□ Percentage increase in cell number. The data were obtained from the same cultures as those of Figs 3 and 4. D1, D2, and D3 indicate the times of the first, second, and third divisions.

(Fig. 2). The r-RNA content per explant remained constant from 22–35 h, i.e. during the first S period, then increased steeply between 35 and 40 h. This second rise in r-RNA content coincided with the second increase in the rate of r-RNA synthesis. After 40 h, the r-RNA content of the explants increased very slowly.

Although the rate of r-RNA synthesis was high during the first S period (Fig. 3), very little net RNA accumulation occurred in this time (Fig. 5). This suggests that the rate of r-RNA turnover may be important in controlling tissue r-RNA content, and that turnover rate may vary with time of culture.

We could detect no decrease in the rate of r-RNA synthesis or accumulation at the time of the first division, although it is now well established that contracted mitotic chromosomes do not serve as templates for RNA synthesis (Das, 1963; Scharff and Robbins, 1965). It is unlikely that any reduction in the rate of r-RNA synthesis in the individual cell would be detected in the explant as a whole: a sharp increase in r-RNA synthesis followed closely after division, and the synchrony of division is not complete. Evans (1967) calculated that division in the

individual cell takes 3 h, in the entire explant 6 h. These two factors could obscure a temporary decrease in the rate of r-RNA synthesis.

The t-RNA content of explants remained constant for the first 35 h after excision, then doubled between 35 and 40 h, i.e. together with the second increase in r-RNA content (Fig. 5).

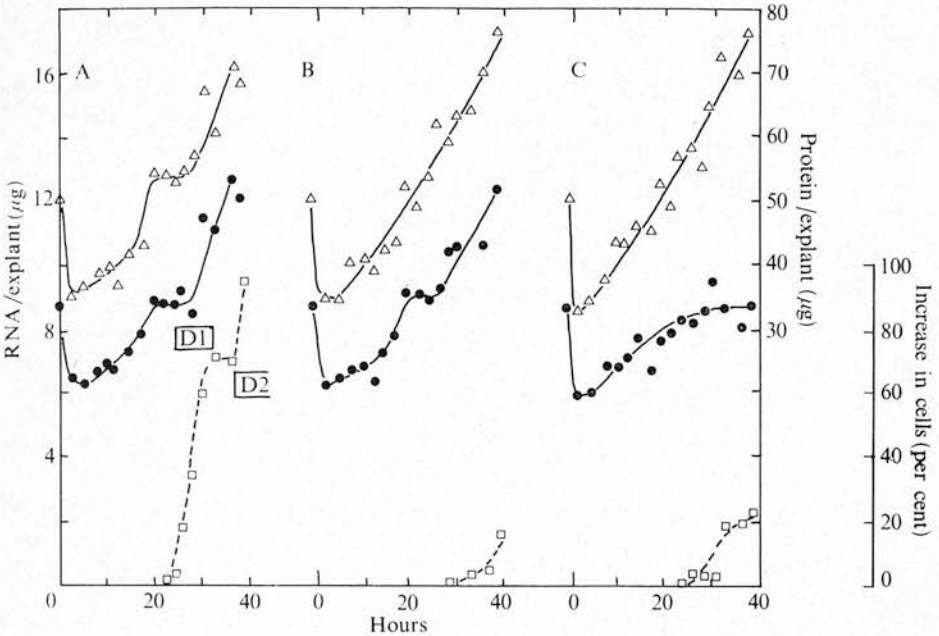

FIG. 6. Changes with time in total RNA (●—●) and protein (△—△) contents, and percentage increase in cell number (□- -□) for A. control explants grown in total darkness; B. explants grown in total darkness in medium containing 1 μg ml^{-1} mitomycin C; C. explants grown under 4 klx mixed tungsten and fluorescent light. D1 and D2 indicate the times of the first and second divisions.

Figure 6 shows changes in total RNA and protein contents of explants which had a short lag period before the first division. The initial loss of RNA and protein was from cells cut or damaged during excision. RNA and protein contents then increased, remained constant during the latter part of S, then increased again at the end of the first division. Thus the accumulation of protein, like RNA, occurred in two distinct periods. Changes in RNA contents in explants with a short lag period before division (Fig. 6) were similar to those in explants with a long lag period (Fig. 5), with one exception: explants with the shorter lag period did not have the phase at the start of culture during which no increase in RNA content occurred.

RNA and protein accumulation in non-dividing explants

To test how many of the changes recorded above were dependent on the simultaneous occurrence of DNA synthesis and cell division, we studied explants in which DNA synthesis and cell division had been prevented by various means.

DNA synthesis, cell division, and RNA accumulation all depended highly on the presence of 2,4-D (Table 1). In contrast, protein accumulation was relatively indepen-

dent of 2,4-D concentration; in the absence of 2,4-D, protein content increased by 80 per cent over 67 h of culture.

Growth of explants under high light intensity inhibits DNA synthesis and cell division (Table 2; Fraser *et al.*, 1967). Protein accumulation was not inhibited by high light intensity. RNA accumulation did occur, but was less than in the dark-grown control explants (Fig. 6). It appeared that the first rise in RNA content occurred in the light, but that the second rise was completely inhibited.

TABLE 1. *Increase in cell number and changes in DNA, RNA, and protein contents of explants grown for 67 h in medium with different concentrations of 2,4-D*

	Hours of culture				
	0	67	67	67	67
2,4-D concentration (M)		0	10^{-8}	10^{-6}	10^{-5}
% Increase in cell number		1	10	96	120
μg DNA/explant	1·21[a]	0·96	1·48	2·30	2·50
μg RNA/explant	5·6	5·4	7·5	13·7	14·2
μg protein/explant	25	44	48	54	57

[a] The DNA, RNA, and protein time O values include material in damaged, surface cells which is lost within the first hour of culture (cf. Fig. 4).

TABLE 2. *Changes in DNA content and increase in cell number of explants grown under different light intensities*

	Hours of culture	μg DNA/explant	% Increase in cells
Light intensity			
0 lx	0	1·2	..
	67	2·4	110
4500 lx	67	1·3	30

The antibiotic mitomycin C inhibited DNA synthesis and cell division (Table 3). Neither RNA accumulation nor protein accumulation was inhibited (Fig. 6). Furthermore, explants grown in mitomycin C accumulated RNA in the same stepwise manner as control explants. It was not possible consistently to demonstrate a stepwise accumulation of protein in mitomycin C or light-treated explants.

TABLE 3. *Changes in DNA content and increase in cell number of explants-grown in medium with various concentrations of mitomycin C*

	Hours of culture	μg DNA/explant	% Increase in cells
μg ml^{-1} mitomycin C			
0	0	1·0	..
0	57	3·9	385
1	57	1·1	15
5	57	0·9	0
20	57	0·9	0

It would have been interesting to look at the patterns of RNA and protein accumulation in explants in which division occurred, but asynchronously. Unfortunately, no experimental conditions are known which will asynchronize cell division. The synchrony of early cell division is inherent, not the result of any applied treatment or selection. It appears to be a consequence of placing similarly endowed cells simultaneously under conditions promoting cell division.

DISCUSSION

Excision and culture of explants induced DNA synthesis, highly synchronous cell division (Fig. 1), and large increases in RNA and protein contents. The accumulations of RNA and protein commenced immediately after excision in explants cut from fresh or young tubers (Fig. 6). In contrast, when explants were taken from old tubers, a period of very low rate of r-RNA synthesis and little r-RNA accumulation followed excision (Figs 3, 5). There was also an increase in the length of the lag period before division with explants from older tubers. As the tuber ages, cells become more committed to breakdown to support bud growth. Explants cut from tubers stored for 6 months had 30 per cent less RNA and protein than those cut from newly harvested tubers, though they contained similar numbers of cells (Fraser, 1968). Thus explants from older tubers seem to require a period of metabolic readjustment before major RNA synthesis can begin. To some extent the 'starting point' for the process of de-differentiation induced by excision and culture can be selected by choosing appropriate tubers. This may facilitate the study of de-differentiation in this system.

During the first two divisions in explants, the increase in metabolic activity was not continuous, but occurred in two distinct periods. In these periods, the rate of r-RNA synthesis (Fig. 3), r-RNA (Fig. 5) and total RNA (Fig. 6) accumulation, protein accumulation (Fig. 6), and phosphate uptake (Fig. 3) all increased. DNA synthesis for the first (Fig. 3) and second divisions commenced during the first and second periods respectively. We have therefore shown that in the early growth of the explants, it is not only cell division which is synchronous; other aspects of metabolism also change in a periodic manner. Evans (1967) and Mitchell (1969), using culture conditions under which about 35 per cent of cells divided, also found stepwise increases in total RNA and protein contents.

Experiments with explants in which cell division and DNA synthesis were prevented showed that some aspects of early metabolism could occur largely independently. In particular, the accumulation of protein was not dependent on a concomitant rise in the RNA content of the explant, i.e. on an increase in the number of ribosomes (Table 1). Both RNA and protein accumulation could occur in explants in which cell division and DNA synthesis had been inhibited (Fig. 6). Furthermore, the RNA accumulation pattern remained stepwise in explants with inhibited DNA synthesis and cell division.

Changes in the rates of r-RNA synthesis and accumulation

In each of the two cell cycles studied, the rate of r-RNA synthesis increased in a stepwise manner. This pattern is superficially similar to some reports of the pattern

of r-RNA synthesis during the cell cycle in mammalian cells (Klevecz and Stubblefield, 1967; Pfeiffer and Tolmach, 1968). In mammalian cells, the step in the rate of r-RNA synthesis occurs during DNA synthesis, and may be caused by the doubling of the number of r-RNA genes. It is unlikely that the number of r-RNA genes was the factor controlling the rate of r-RNA synthesis in artichoke explants. The first increase in the rate of r-RNA synthesis occurred before the beginning of DNA synthesis in the explant (Fig. 3). RNA accumulation was uninhibited and remained stepwise when DNA synthesis was inhibited by mitomycin C (Fig. 6). In dividing explants, the rate of r-RNA synthesis remained constant during S phase, when the r-RNA gene number would be expected to double. These results suggest that the rate of r-RNA synthesis was controlled by the activity of genes, not their number. Also, the pattern of changing rate of r-RNA synthesis cannot be related through gene-number effects to the cell cycle rhythm in the tissue. What then was the cause of the stepwise pattern of early RNA metabolism?

Circumstantial evidence favours the following interpretation: The first increase in r-RNA synthesis is stimulated by factors related to excision and damage to the outer cells of the explant. When explants were grown without 2,4-D, a small rise in RNA content occurred at the time of the first increase in control explants grown with 2,4-D. The size of this small rise in RNA was increased by adding homogenized explants to the medium (Fraser, 1968). Excision and surface damage are also known to stimulate RNA synthesis in potato tuber tissue (Sampson and Laties, 1968). The second rise in RNA content seems entirely dependent on 2,4-D. The succession of the early, transitory, excision-related stimulus to RNA synthesis, and the later stimulation by 2,4-D, could produce a stepwise pattern of development of metabolic activity and RNA synthesis.

The conclusion from this study is that much of early metabolism is synchronous; the synchrony of cell division is merely one manifestation of this. The pattern of cell number increase alone might suggest that this system would be a good one to use in studies of events related to the cell cycle. This is not entirely the case, as we have shown that major early metabolic changes occur independently of cell division, and periodic changes need not be related temporally to a cell cycle rhythm. Further, the first division, which is the most synchronous, must be considered unusual as it includes a very extended G_1. The main use of the system should be in the study of induction of cell division in previously non-dividing cells, and the resulting de-differentiation. A later paper will discuss the synthesis of RNA necessary for the induction and continuance of cell division.

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Printed in Great Britain
by William Clowes & Sons Limited
London, Colchester and Beccles

Reprinted from
Journal of Experimental Botany
Vol. 26, No. 93

Oxford : at the Clarendon Press

Journal of Experimental Botany, Vol. 26, No. 93, pp. 555–568, August 1975

Synchronous Cell Division in Cultured Explants of Jerusalem Artichoke Tubers: the Effects of 5-Fluorouracil on Messenger RNA Synthesis and the Induction of Cell Division

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Received 2 January 1975

ABSTRACT

Explants of secondary xylem parenchyma tissue from Jerusalem artichoke tubers were induced to undergo cell division and de-differentiate by culture in nutrient medium. The first division was inherently synchronous. The system was used to study the involvement of messenger RNA synthesis in the induction and continuance of cell division in previously non-dividing cells.

The base analogue 5-fluorouracil (5-FU) inhibited ribosomal RNA synthesis and the processing of ribosomal RNA precursor to mature 25 S and 18 S RNAs. The synthesis of messenger-like RNAs (heterogeneous in size, labelled to a high specific activity in a pulse incubation, and containing a polyadenylic acid sequence) was less inhibited by 5-FU.

Explants grown in 5-FU did not synthesize DNA and did not divide. A direct inhibition of DNA synthesis by 5-FU added late in culture was reversed by thymidine. An indirect inhibition of DNA synthesis occurred when 5-FU was present from the start of culture and was not reversed by thymidine. Because ribosomal RNA synthesis is not necessary for the induction of cell division (Fraser, 1975) and because 5-FU was incorporated into mRNA, probably interfering with its function, these results suggest that 5-FU inhibited the metabolism of mRNA which was required for DNA synthesis and cell division.

The timing of mRNA synthesis required for DNA synthesis and cell division was investigated by adding 5-FU plus thymidine to cultures at various times. By the beginning of DNA synthesis for the first division, explants were competent, in terms of mRNA synthesized, to complete the first division. Messenger RNA synthesis occurring before the end of the first division allowed explants to undergo at least three more divisions.

INTRODUCTION

When explants of tissue from the quiescent tuber of Jerusalem artichoke are cultured in nutrient medium with the synthetic auxin 2,4-dichlorophenoxyacetic acid, vigorous cell division is induced (Yeoman, Dyer, and Robertson, 1965; Fraser, Loening, and Yeoman, 1967). By repeated divisions, the large, highly vacuolated storage parenchyma of the original explant de-differentiates to small, meristematic-type cells. Several features of this tissue culture system make it suitable for the study of the transition of cells from one developmental state to

another. The tissue is highly uniform, consisting almost entirely of one type of cell. In culture, 80–90 per cent of cells undergo division (Fraser *et al.*, 1967). The first few divisions are inherently synchronous (Yeoman and Evans, 1967). Other aspects of metabolism, such as ribosomal RNA (rRNA) synthesis, protein synthesis, and phosphate uptake also change in a periodic manner during culture (Fraser and Loening, 1974).

Cell division in explants commences after a lag of between 20 and 60 h; the duration of the lag period increases with length of storage of the tubers. Before cells divide, they have to synthesize DNA. Mitchell (1967) has shown that DNA synthesis (S phase) for the first division is reproducibly around 12 h long. Variation in the total length of the pre-division lag period is a result of variation in the length of the G1 period, between excision and the beginning of S. G2, the period between the end of S and mitosis, is 0–2 h long.

RNA metabolism is likely to play a controlling role in the induction of DNA synthesis and cell division. The freshly excised explant contains both rRNA (Fraser and Loening, 1974) and polyadenylated, messenger-like RNA (Fraser, 1975). During the first few hours of culture, the predominant types of RNA synthesized are messenger-like. A few hours before the beginning of DNA synthesis for division 1, the rate of rRNA synthesis increases five-fold. Explant content of rRNA has almost doubled by the time of the first division (Fraser and Loening, 1974). However, these increases in rRNA content and synthesis rate are not required for the occurrence of the first division. Low levels of actinomycin D inhibit rRNA synthesis much more than mRNA synthesis in artichoke explants as in other tissues. Explants grown with low levels of actinomycin D do not synthesize rRNA but do complete the first division (Fraser, 1975).

This paper reports studies on the requirement for mRNA synthesis for the induction and continuance of cell division. The simplest approach to this problem would be to inhibit all RNA synthesis and investigate the effects on cell division. Actinomycin D cannot be used for this purpose, as even high concentrations do not give immediate inhibition of mRNA synthesis (Fraser, 1975). High actinomycin D concentrations may also inhibit DNA synthesis directly (Reich and Goldberg, 1964).

The base analogue 5-FU was chosen as an alternative means of interfering with RNA metabolism. The incorporation of 5-FU into mRNA in bacteria interferes with mRNA function in protein synthesis (reviewed by Heidelberger, 1963; 1965) and 5-FU is known to be incorporated into plant RNA (Key, 1966). The effects of 5-FU on cell division and DNA, RNA, and protein synthesis in artichoke explants were investigated. Use was made of the recent finding that many eukaryote mRNAs contain a polyadenylic acid sequence (Edmonds, Vaughan, and Nakazato, 1971; Higgins, Mercer, and Goodwin, 1973) to isolate an mRNA fraction for study. The results suggest that while 5-FU did not drastically inhibit mRNA synthesis, it inhibited DNA synthesis and cell division by interfering with the function of mRNAs required for these events. 5-FU was used to determine the times by which explants were competent, in terms of mRNA synthesized, to complete the following cell division.

MATERIALS AND METHODS

Culture conditions

Explants, 2·5 mm long, 2 mm diameter, 9 mg fresh weight, and containing 20 000 cells, were removed aseptically from the secondary xylem storage parenchyma tissue of Jerusalem artichoke (*Helianthus tuberosus* L. var. 'Bunyard's Round') tubers. Thirty explants were cultured in 5·25 ml medium containing salts (Bonner and Addicott, 1937), 4 per cent (w/v) sucrose, and 10^{-5} M 2,4-dichlorophenoxyacetic acid. Cultures were grown in 90-mm-diameter petri dishes, agitated at 50 cycles min^{-1} on a reciprocating shaker with a 50 mm displacement. The explants were excised in dim green light, and cultured in total darkness at 25 °C (Fraser *et al.*, 1967). 5-FU and thymidine were dissolved in culture medium at ten times the required final concentration, and one-tenth volume was added to cultures through a Millipore filter.

Radioactive incubations

[8-^{3}H]adenine (10 Ci mol^{-1}), [6-^{3}H]5-FU (1 Ci mol^{-1}), and [^{32}P]orthophosphate (84 Ci mg^{-1} P) were obtained from the Radiochemical Centre, Amersham. [^{3}H]adenine and [^{3}H]5-FU were added directly to cultures to concentrations of 80 μCi ml^{-1}. For ^{32}P incubations, explants were transferred to phosphate-free culture medium and labelled with 85 μCi ml^{-1} ^{32}P for 15 min. Explants were then returned to phosphate-containing non-radioactive culture medium for a 'chase' of 75 min.

Measurement of DNA synthesis

Fifteen explants labelled for 1 h with 2 μCi ml^{-1} [^{3}H]adenine were weighed, and then homogenized in 5 ml 5 per cent (w/v) trichloroacetic acid (TCA) at 0 °C. The precipitate was washed three times with 5 per cent TCA and three times with 80 per cent (v/v) ethanol containing 0·2 M sodium acetate, pH 6·0. The precipitate was then extracted for 12 h with 5 ml 1 M KOH at 37 °C to hydrolyse RNA. Perchloric acid (PCA) was added to reduce the pH to 2, and the precipitate was collected by centrifugation and washed twice with cold 5 per cent (v/v) PCA. DNA was then extracted into 2 ml 5 per cent (v/v) PCA for 45 min at 70 °C. Aliquots of the extract were counted for radioactivity in butyl-PBD:toluene:methoxyethanol (0·5g: 60 ml: 40 ml) scintillator. A control experiment showed that over 90 per cent of the radioactivity not rendered soluble in PCA by KOH extraction was digestible by DNase.

Extraction of nucleic acids

Nucleic acids were extracted from labelled explants by a detergent-phenol procedure and further purified as explained earlier (Fraser and Loening, 1974). The total yield of nucleic acid was measured by hydrolysing one tenth of the product in 2 ml 5 per cent PCA at 70 °C for 20 min. The ultraviolet absorption spectrum was measured in the SP 800 recording spectrophotometer and RNA concentration calculated from the absorbance at 260 nm.

Fractionation of RNA on oligo(dT)-cellulose

Extracted RNA was fractionated into poly(A)-containing and non-poly(A)-containing fractions using the selective binding at high salt concentration of polyadenylated RNA to oligodeoxythymidylic acid-cellulose (Edmonds and Caramela, 1969; Aviv and Leder, 1972). Between 50 and 100 μg of total nucleic acid were dissolved in 1 ml binding buffer (400 mM NaCl; 10 mM Tris-HCl, pH 7·8; 1 mM EDTA; 0·1 per cent sodium dodecyl sulphate) and shaken with 20 mg oligo(dT)-cellulose (Collaborative Research Inc., Waltham, Mass, U.S.A.) for 30 min at 20 °C. The cellulose was sedimented at 12 000 *g* for 1 min and washed four times with 0·5 ml portions of binding buffer. All binding buffer supernatants were combined and the DNA and those RNAs lacking poly(A) were precipitated by 2·5 vol. ethanol at 0 °C. The cellulose was then washed four times with 0·25 ml elution buffer (binding buffer minus NaCl). Poly(A)-containing RNA was precipitated from the combined elution buffer washes by addition of 50 μg non-radioactive artichoke carrier RNA, NaCl to 0·3 M, and 2·5 vol. ethanol.

Sucrose gradients

RNA (25–50 μg) was dissolved in 0·1 ml gradient buffer (50 mM Tris-HCl, pH 7·8; 150 mM NaCl; 1 mM EDTA; 0·5 per cent sodium dodecyl sulphate) and layered on to a 5 ml linear gradient of 5–25 per cent (w/v) sucrose in gradient buffer. Gradients were centrifuged for 2 h at 50 000 rev. min^{-1} (234 000 *g* at r_{av}) at 25 °C, in the SW 50·1 rotor of the Spinco Model L

centrifuge. Gradients were continuously monitored for absorbance at 260 nm by upward displacement through a flow cell fitted to a Joyce-Loebl Gel Scanner. Between 36 and 40 fractions were collected, and RNA was precipitated by addition of 200 μg bovine serum albumin as carrier and 1 ml 10 per cent TCA. The precipitated RNA was collected by filtration on squares of Whatman GF/A glass fibre paper and washed three times with 5 ml cold 10 per cent TCA. The filters were dried, immersed in 5 ml 0·5 per cent butyl-PBD/toluene scintillator and [^{3}H]radioactivity was determined in a liquid scintillation counter.

Polyacrylamide gel electrophoresis

^{32}P-labelled RNA (20 μg) was fractionated on a 2·4 per cent polyacrylamide gel (Loening, 1967) for 2 h at 8 V cm^{-1} gel length. The gels were frozen and sliced into 0·5-mm-thick slices, and radioactivity in each slice was determined in a Beckman Lowbeta counter. Total radioactivity associated with rRNA precursor (Rogers, Loening, and Fraser, 1970), 25 S plus 18 S rRNAs, and polydisperse RNA were determined from the radioactivity profile.

Total DNA, RNA, and protein contents

Determinations were performed in triplicate on samples of ten explants. Removal of contaminants was by the method of Holdgate and Goodwin (1965). RNA was measured by extinction at 260 nm after extraction by the Schneider (1945) procedure, DNA by the diphenylamine method (Burton, 1956), and protein by the method of Lowry, Rosebrough, Farr, and Randall (1951). Full details of the assay methods are given elsewhere (Fraser and Loening, 1974).

Cell number

Five explants were macerated for 24 h at 20 °C in 2 ml 5 per cent (w/v) chromic acid (Brown and Rickless, 1949). This treatment does not degrade the wall of the original cell; thus cells arising from division of a single original cell of the explant are found together within the parental cell wall in the macerate. By scoring a sample of macerate for single cells, pairs (first division products), fours (second division products), etc., it was possible to calculate the percentage increase in cell number which had occurred during culture. Further, the ability to recognize products of particular divisions made it possible to determine accurately the timing of each division, and to determine which divisions had contributed to the increase in cell number in the explant.

Detailed descriptions of the counting method and the pattern of cell divisions, together with evidence for the synchrony of the first division, are given in Fraser and Loening (1974).

Measurement of total RNA synthesis

Nucleic acids were extracted from explants labelled with [8-^{3}H]adenine or [6-^{3}H]5-FU by the detergent-phenol procedure (Fraser and Loening, 1974). RNA synthesis was estimated by the incorporation of [^{3}H]radioactivity into TCA-insoluble material. DNA was removed by DNase treatment (Loening, 1967). RNA was precipitated from the extract by addition of TCA to a final concentration of 10 per cent. The precipitate was collected by filtration on glass fibre paper and washed three times with cold 10 per cent TCA, and [^{3}H]radioactivity on the filter was determined as above.

RESULTS

Effects of 5-FU on RNA synthesis

Fig. 1 shows the effects of 5-FU on RNA synthesis in artichoke explants. Cultures, to which 5-FU had been added at various times, were labelled with [^{32}P]orthophosphate from 22–23·5 h after excision. The amount of ^{32}P taken up into the alcohol-soluble pool (Fraser and Loening, 1974) was similar in control and 5-FU-treated explants. Differences in the incorporation of ^{32}P into RNA species in control or 5-FU-treated explants are therefore likely to have arisen by different rates of RNA synthesis rather than from different isotope uptake rates.

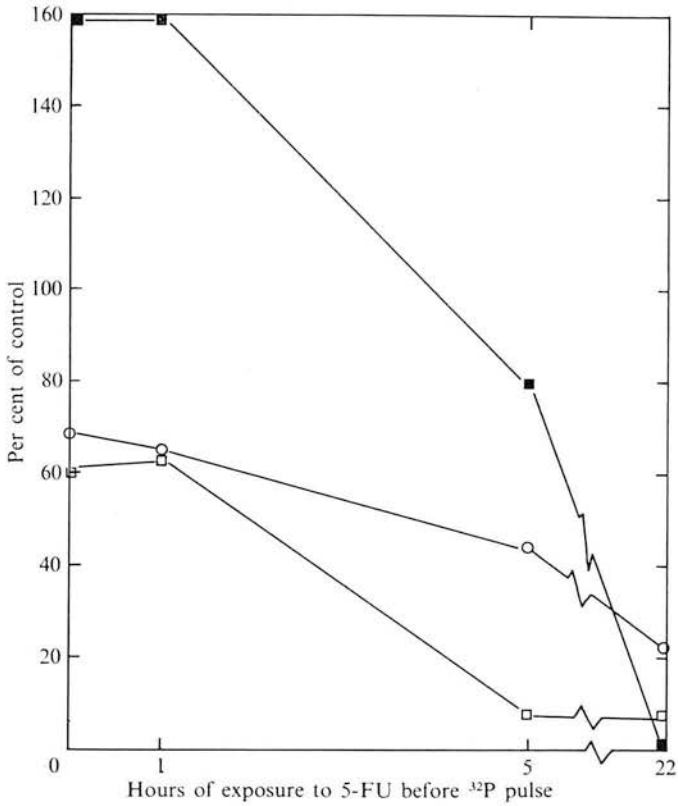

FIG. 1. Incorporation of ^{32}P into rRNA precursor (■——■), 25 S plus 18 S mature rRNA (□——□), and polydisperse RNA (○——○) in artichoke explants labelled from 22–23·5 h.

1 mM 5-FU was added to cultures at various times before the start of labelling. Results are expressed as percentages of labelling in control explants to which no 5-FU was added. Absolute amounts of ^{32}P incorporation in control explants were (in 10^3 ct min^{-1} per explant) rRNA precursor: 2·5; 18 S plus 25 S rRNA: 25; polydisperse RNA: 45.

18 S and 25 S rRNA labelling was immediately 40 per cent inhibited by 5-FU, and was almost eliminated by 5 h exposure to the drug. In explants treated with 5-FU for 0 or 1 h prior to labelling, there was considerably more radioactivity in the rRNA precursor than in control explants. This suggests that 5-FU inhibited the processing of precursor rRNA to mature 18 S and 25 S molecules. A similar inhibition of rRNA precursor processing was shown in mammalian cells by Willen and Strenram (1967). In the experiment shown in Fig. 1, rRNA precursor was labelled one tenth as much as mature rRNAs in control explants. Thus, despite the accumulation of labelled rRNA precursor in 5-FU-treated explants, the overall effect of 5-FU on rRNA synthesis was an inhibition.

The synthesis of polydisperse, messenger-like RNA was least affected by 5-FU. Even after 22 h exposure, the synthesis of polydisperse RNA was 20 per cent of the control level.

The effects of 5-FU on mRNA synthesis were examined in more detail by labelling explants during the first 3 h of culture, when rRNA synthesis is low (Fraser and Loening, 1974) and the synthesis of messenger-like RNAs predominates (Fraser,

1975). RNA extracted from explants after radioactive incubation was separated into two fractions, one with and one without a polyadenylic acid sequence, using oligo(dT)-cellulose (Edmonds and Caramela, 1969; Aviv and Leder, 1972).

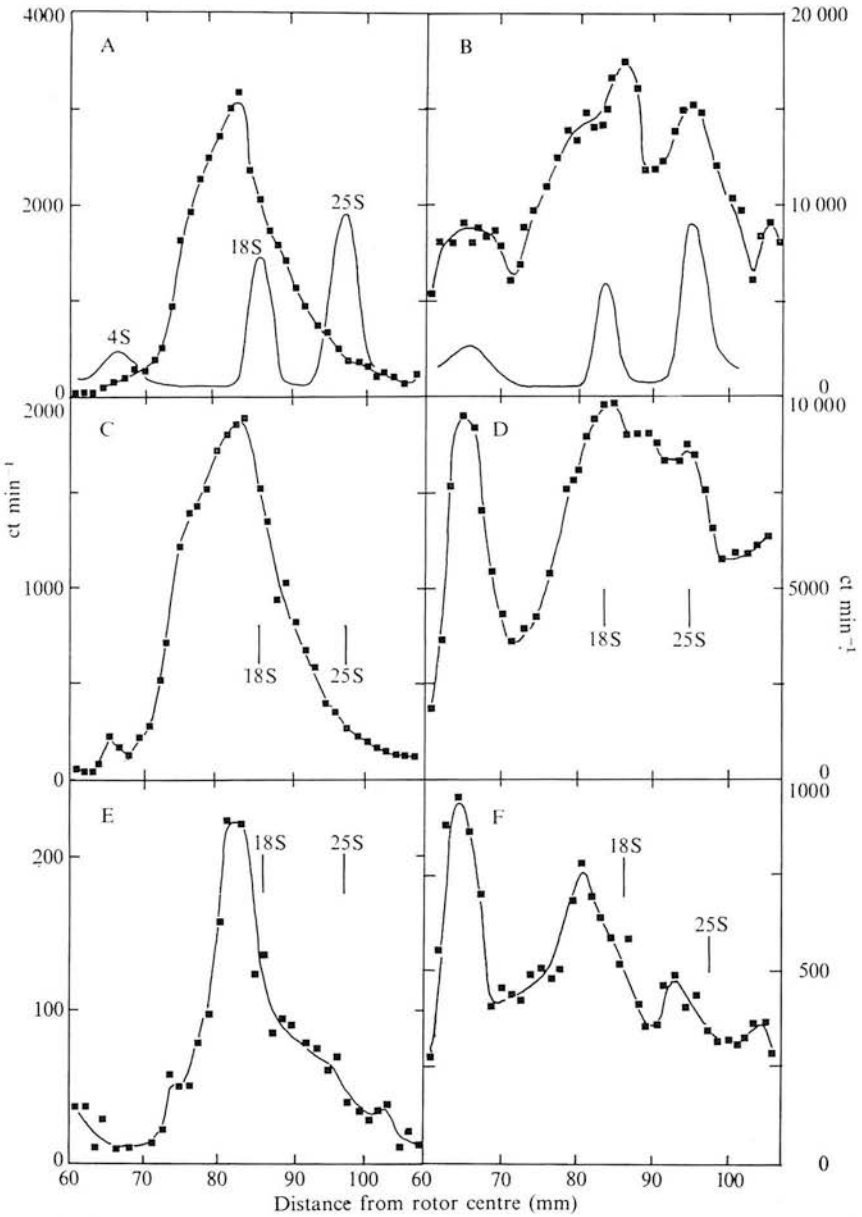

FIG. 2. Sedimentation in sucrose density gradients of labelled RNA from artichoke tuber explants.

Radioactivity (■——■); absorbance at 260 nm (——). A, C, and E show polyadenylated RNA fractions prepared by oligo(dT)-cellulose binding. B, D, and F show the non-poly(A)-containing RNAs which did not bind to oligo(dT)-cellulose at high salt concentration. A and B: RNAs from control explants labelled with [8-^{3}H]adenine from 0·5–2·5 h. C and D: RNAs from explants cultured with 1·0 mM 5-FU from 0 h and labelled with [8-^{3}H]adenine from 0·5–2·5 h. E and F: RNAs from explants labelled with [6-^{3}H]5-FU from 0–2 h. All counts are expressed per 100 μg total RNA fractionated on oligo(dT)-cellulose. The positions of the 18 S and 25 S rRNA absorption peaks are shown by vertical lines in the lower panels.

In control explants labelled with [^{3}H]adenine from 0·5–2·5 h, about 15 per cent of the radioactivity incorporated into RNA was in the poly(A)-containing RNA (Fig. 2A). This RNA was of high specific activity, and sedimented on a sucrose gradient to give a polydisperse distribution from 6 S to 30 S, with a mean around 16 S. Fig. 2B shows the non-poly(A)-containing RNAs synthesized in control explants. Radioactivity peaks were associated with the optical density peaks for transfer RNA and 18 S and 25 S rRNAs, but most of the incorporation was into polydisperse RNA with sedimentation coefficients from about 7 upwards.

When explants were grown with 1 mM 5-FU from 0 h and labelled with [^{3}H]-adenine from 0·5–2·5 h, the radioactivity of the poly(A)-containing RNA was 60 per cent of that in the control (Fig. 2C and A). The size distribution of the poly(A)-containing RNA was the same in control and 5-FU-treated explants. There is some evidence that larger poly(A)-containing RNAs may be precursors of smaller polyadenylated RNAs in artichoke tissue (Fraser, 1975 and unpublished data). The results of Fig. 2C suggest that 5-FU did not inhibit the processing of large to small polyadenylated molecules. This is in contrast to the effects of 5-FU on processing of the rRNA precursor (Fig. 1 and Willen and Strenram, 1967).

The labelling of non-poly(A) RNAs in 5-FU-treated explants (Fig. 2D) was also overall about 60 per cent of the control level (Fig. 2B) but there were major qualitative differences in the types of RNA synthesized in control and treated explants. Labelling of 18 and 25 S rRNAs in treated explants was barely detectable, but the synthesis of the non-polyadenylated polydisperse RNA was prominent. A sharp peak of radioactivity was present in the 4 S region in gradients of RNA from 5-FU-treated explants. This low molecular weight component appears similar to an RNA of between 4 and 5 S isolated from 5-FU-treated *Trichoderma* by Gressel and Galun (1966).

In sum, the data of Figs 1 and 2 show that 5-FU inhibited rRNA synthesis most severely, but had less effect on the synthesis of the polyadenylated and non-polyadenylated polydisperse RNA fractions. These two polydisperse RNA fractions had two features characteristic of messenger RNAs; they were labelled to a high specific activity in a pulse incubation, and were heterogeneous in size. Poly(A)-containing RNA is known to be associated with the polyribosomes in plants (Higgins, Mercer, and Goodwin, 1973). The poly(A)-containing RNA described here probably included cytoplasmic mRNA. The role of the non-poly(A)-containing polydisperse RNA is not clear. It may have included cytoplasmic mRNA, as examples are known of non-polyadenylated polyribosomal mRNAs in other eukaryotes (Adesnik and Darnell, 1972; McLaughlin, Warner, Edmonds, Nakazato, and Vaughan, 1973; Fraser, 1973).

These findings on the effects of 5-FU on RNA synthesis confirm and extend the results of Key (1966), who found that 5-FU inhibited the synthesis of rRNA more than that of dRNA (RNA of DNA-like base composition) in soybean hypocotyl.

Incorporation of 5-FU into RNA

RNAs labelled when explants were incubated with [^{3}H]5-FU from 0–2 h are shown in Fig. 2E and F. [^{3}H]5-FU was incorporated into the polyadenylated RNA

(Fig. 2E). The RNA synthesized was polydisperse, from 6 to 30 S, and, like the [^{3}H]adenine-labelled RNA of control explants (Fig. 2A), had a mean sedimentation coefficient of 16 S. A high proportion of the [^{3}H]5-FU-labelled poly(A)-containing RNA molecules had a sedimentation coefficient very close to the mean.

[^{3}H]5-FU was incorporated into the sharp, low molecular weight RNA peak in the non-poly(A) fraction (Fig. 2F), and also into two peaks sedimenting rather slower than the 18 and 25 S rRNA optical density peaks. In bacteria, rRNA which contains 5-FU has been shown to form incomplete ribosomal subunits; the rRNA is quickly degraded (Andoh and Chargaff, 1965). It is likely that the two radioactivity peaks in Fig. 2F were similar degradation products of 5-FU-containing rRNA. [^{3}H]5-FU also appeared to be incorporated into the non-poly(A) polydisperse RNA (Fig. 2F). Key (1966) found incorporation of 5-FU into rRNA, tRNA, and dRNA in soybean hypocotyls.

5-FU and DNA synthesis

5-FU inhibits DNA synthesis directly; some 5-FU is converted in the cell to 5-fluorodeoxyuridylic acid, which inhibits the enzyme thymidylate synthetase, leading to a shortage of thymidylic acid, hence an inhibition of DNA synthesis. In bacteria and mammalian cells, the inhibition of DNA synthesis can be reversed by thymidine (Cheong, Rich, and Eidinoff, 1960; Horowitz, Saukkonen, and Chargaff, 1960; Paul and Hagiwara, 1962; Heidelberger, 1963). The effects of 5-FU and thymidine on DNA synthesis in artichoke explants were investigated.

Explants were grown for 47 h (about the middle of the first S phase in the tissue used) before addition of 5-FU (0·1 mM) with and without thymidine (0·33 mM). This concentration of thymidine was chosen as it is 100 times the amount of thymidine calculated necessary for DNA synthesis for division 1 in the culture, but is much lower than the concentration of thymidine used to inhibit DNA synthesis in thymidine-block synchronization of mammalian cells (Xeros, 1962; Rao and Engelberg, 1966).

Explants were labelled with [^{3}H]adenine from 49–50 h, and incorporation of isotope into DNA was determined (Table 1). 5-FU alone inhibited DNA synthesis by about 80 per cent. Thymidine alone had no effect. In explants treated with 5-FU plus thymidine, DNA synthesis was similar to the level in control explants. Thus, in artichoke explants, thymidine reverses the inhibition of DNA synthesis by 5-FU.

5-FU and explant growth

5-FU, with and without thymidine, was added to explants at the start of culture, and changes in cell number and DNA, RNA, and protein contents were followed (Fig. 3). 5-FU completely inhibited cell division and RNA and DNA accumulation, and thymidine gave no reversal of inhibition of any parameter. Protein accumulation was reduced to about 40 per cent of the level in control explants by 5-FU with or without thymidine.

The important difference between the results of Table 1 and Fig. 3 is that, in the presence of thymidine, 5-FU did not inhibit DNA synthesis when added in the middle of S phase (at 47 h in Table 1), but did inhibit DNA synthesis when present

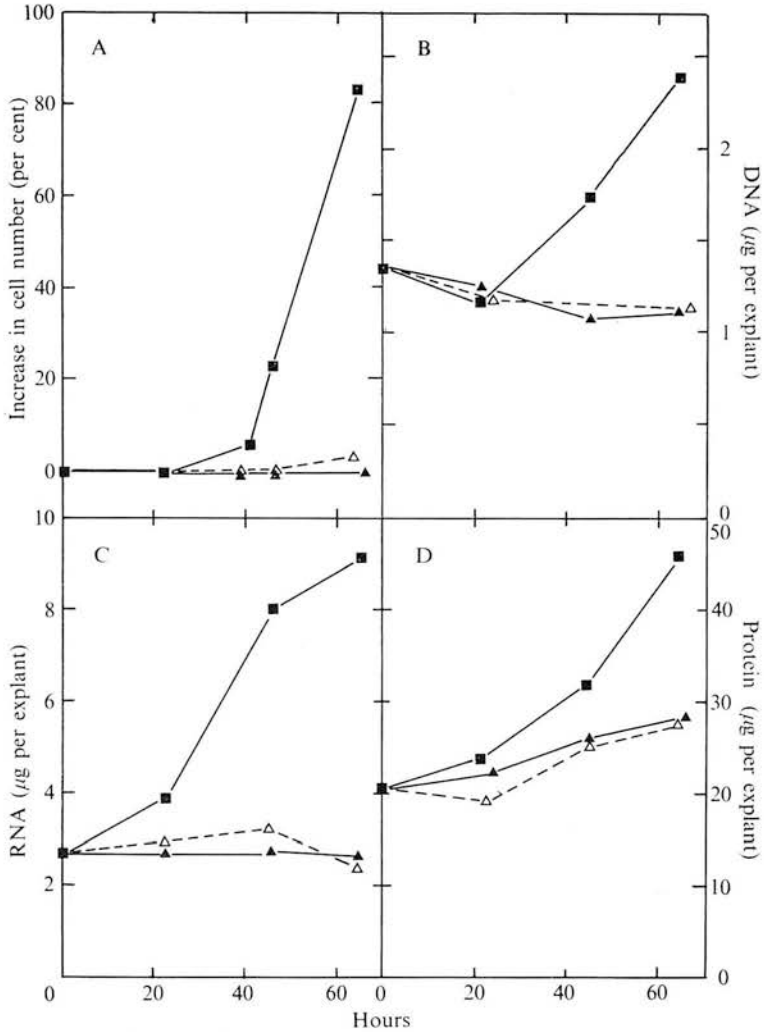

FIG. 3. Percentage increase in cell number (A), changes in explant content of DNA (B), RNA (C), and protein (D) during growth in control explants (■——■), and in explants grown with 0·5 mM 5-FU (▲——▲) or 0·1 mM 5-FU plus 0·33 mM thymidine (△---△).

TABLE 1. *Incorporation of [8-^{3}H]adenine into DNA in artichoke tuber explants*

Various additions of 5-FU (0·1 mM) and thymidine (0·33 mM) were made to cultures at 47 h; radioactive incubation was from 49–50 h.

Treatment	[8-^{3}H]adenine incorporated into DNA (ct min^{-1} g^{-1} fr. wt.)
Control	545
5-FU	114
Thymidine	535
5-FU plus thymidine	640

from the start of culture. This suggests that, sometime early in culture, there is 5-FU-sensitive metabolism which is necessary for DNA synthesis; the inhibition of this process by 5-FU is not reversed by thymidine. The timing of the 5-FU-sensitive process required for DNA synthesis was investigated by adding 5-FU,

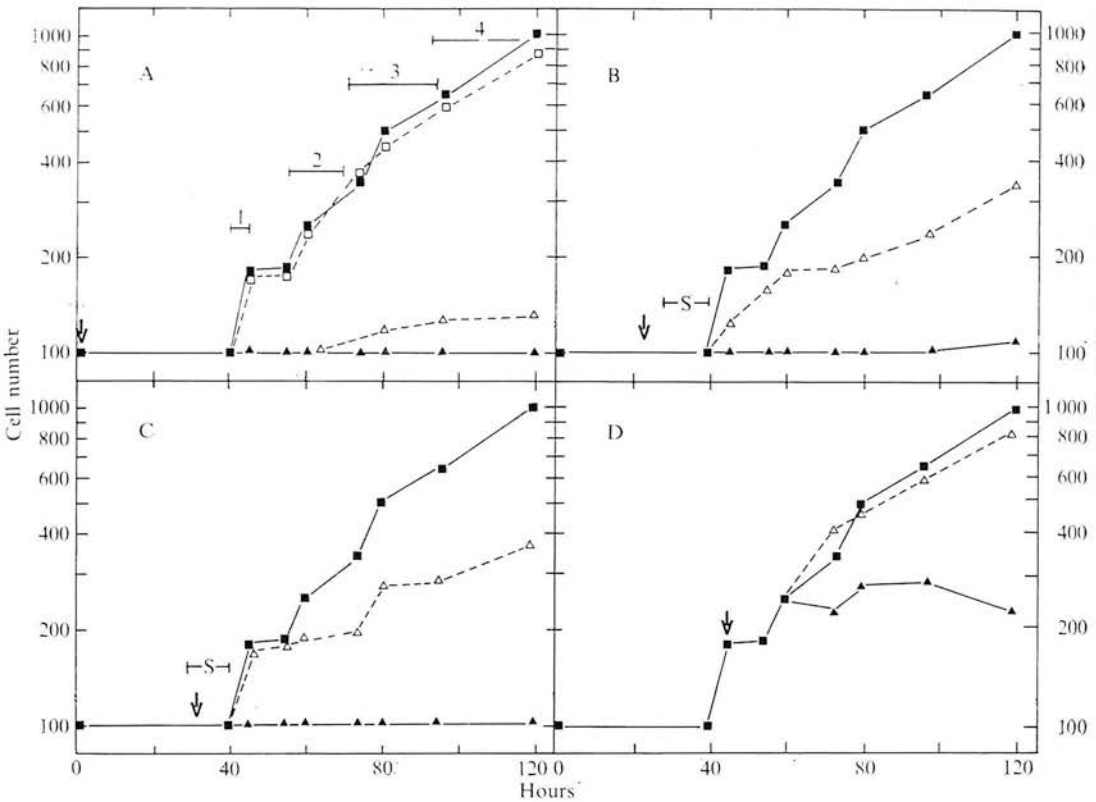

FIG. 4. Changes in cell number (relative to base 100 at 0 h) in control explants (■——■) and explants grown with 0·33 mM thymidine (□---□), 0·1 mM 5-FU (▲——▲), or 0·1 mM 5-FU plus 0·33 mM thymidine (△---△).

Thymidine and 5-FU were added to cultures at 0 h (A), 23 h (B), 31 h (C), or 45 h (D). Vertical arrows indicate the time of addition. The horizontal bars and figures 1–4 indicate the times of the first to fourth cell divisions in control explants, determined by analysis of chromic acid macerates of explants for the appearance of characteristic division products (Fraser and Leoning, 1974). 'S' shows the time of DNA synthesis for division 1, calculated from the data of Mitchell (1967).

with or without thymidine, to cultures after various periods of growth, and following the effects on cell division (Fig. 4).

In the explants used, the first division occurred between 40 and 45 h and the second from 55 to 70 h. DNA synthesis for the first division was calculated from the results of Mitchell (1967) to have been from 28–40 h.

As in Fig. 3, addition of 5-FU at the start of culture completely inhibited cell division. About 25 per cent of the cells eventually divided when thymidine was added with the 5-FU, but the rate of division was very much less than in control explants. Explants to which thymidine alone was added showed the same rate of cell division as control explants.

5-FU added alone at 23 h, before the beginning of DNA synthesis, again completely inhibited cell division. But when thymidine was added with the 5-FU, the first division occurred fully, though at a slower rate than in control explants (Fig. 4B). Thereafter, there was a very slow increase in cell number in the 5-FU plus

thymidine-treated explants, and by the end of the experiment at 120 h just over half of the cells had completed the second division.

5-FU added at 31 h, just after the onset of S phase, completely inhibited the first division. Thymidine added together with the 5-FU permitted the first division to occur exactly as in control explants. However, thereafter the rate of cell division was much lower than in control explants, and again just over half the cells had completed the second division by 120 h.

Addition of 5-FU at 45 h, at the end of the first division, permitted a little second division before complete inhibition of cell number increase. The presence of thymidine again reduced the extent of inhibition of cell division by 5-FU; in this case cell number increased at almost the same rate as in control explants, and by the end of the experiment had risen to five times the value at the time of addition of 5-FU and thymidine.

DISCUSSION

5-FU inhibited the synthesis of DNA in cultured artichoke tuber explants (Table 1 and Fig. 3). The direct inhibition of DNA synthesis was reversed by thymidine when 5-FU and thymidine were added during S phase (Table 1) but not when 5-FU and thymidine were added at the start of culture (Fig. 3). It is concluded that 5-FU added at the start of culture inhibited DNA synthesis indirectly, by affecting some other process required for the occurrence of DNA synthesis. This 5-FU-sensitive process was probably RNA metabolism.

5-FU plus thymidine completely inhibited total RNA accumulation (Fig. 3). However, it is known from experiments with low levels of actinomycin D that a net increase in rRNA is not required for the occurrence of the first division (Fraser, 1975). 5-FU did not completely inhibit the synthesis of those RNAs with messenger-like characteristics (Figs 1 and 2), and protein synthesis was not completely inhibited. However, at least some of the protein synthesized may have resulted from translation of messenger-like RNA which is present in freshly excised explants (Fraser, 1975).

There is considerable evidence from other organisms that incorporation of 5-FU into RNA causes the production of aberrant proteins (Noano and Gros, 1960; Champe and Benzer, 1962; Hignett, 1964), probably by causing errors in base-pairing at either the transcriptional or translational levels. Nakada and Magasanik (1964) were able to show that the induction of enzymically incompetent β-galactosidase in *Escherichia coli* in the presence of 5-FU depended on the incorporation of 5-FU into mRNA. Fig. 2E shows that 5-FU was incorporated into the polyadenylated, polydisperse, messenger-like RNA of artichoke explants. It is therefore suggested that the inhibition of DNA synthesis and cell division by 5-FU plus thymidine, when supplied at the start of culture, was a consequence of interference with mRNA function by incorporated 5-FU, leading to an inhibition of protein synthesis necessary for division, or the synthesis of enzymically incompetent proteins.

On the basis of this mode of action of 5-FU, the data of Fig. 4 may be interpreted to show the time at which cells become competent, in terms of mRNA synthesized,

to complete the following division. The term 'transition point' (Mitchison, 1971) will be used for the time at which competence is attained.

The failure of 5-FU plus thymidine added early in the first S phase to inhibit the first division shows that all mRNAs required for DNA synthesis and cell division had been synthesized by early in S phase (Fig. 4C). The complete, though considerably delayed, first division resulting when 5-FU plus thymidine was added before the beginning of S (Fig. 4B) further shows that much of the required mRNA synthesis had occurred by late G1. These data therefore establish an mRNA transition point for the first division at close to the start of S phase. Experiments with mammalian cells have shown the existence of two RNA transition points, one in G1 for DNA synthesis (Baserga, Estensen, and Petersen, 1965; Frankfurt, 1968) and a second in G2 for cell division (Kishimoto and Lieberman, 1964; Buck, Granger, and Holland, 1967; Doida and Okada, 1969). The occurrence of the mRNA transition point for cell division in late G1—early S in artichoke cells, rather than in G2 as in mammalian cells, may be connected with the comparatively brief G2 in artichoke cells undergoing the first division (Mitchell, 1967). In mammalian cells, G2 occupies a much larger proportion of the cell cycle (reviewed by Mitchison, 1971).

In this study, the question of whether an mRNA transition point for DNA synthesis was located before or with that for cell division was not investigated directly. An mRNA transition point for DNA synthesis must exist somewhere in G1, as cells to which 5-FU plus thymidine was added at 0 h completely failed to synthesize DNA (Fig. 3). Harland, Jackson, and Yeoman (1973) found that the activities of three enzymes required for DNA synthesis all increased at the start of S phase. The simplest interpretation of these results is that mRNA synthesis for these enzymes occurs in the immediately pre-S period, suggesting an mRNA transition point for DNA synthesis close to that for cell division.

In the explants to which 5-FU plus thymidine was added in late G1 or early S, the second division was very much slower than in control cells. Thus the competence acquired by cells in early S to complete division 1 does not extend to allow a normal second division. This could arise in two ways. Firstly, the amounts of mRNA and enzymes per cell are necessarily halved by the occurrence of the first division. Secondly, mRNAs and enzymes may be unstable; those synthesized before the first division may have been largely degraded by the time they are required for the second division. There is direct experimental evidence for this second possibility. Harland *et al.* (1973) found that the activity per explant of thymidine kinase rose during most of the first S phase, then declined. The activity per cell of thymidine kinase fell before and during the first division to the same low level which is found in freshly excised explants.

When 5-FU and thymidine were added at the end of the first division (Fig. 4D), the cells were able to complete not only the second division but at least the third and fourth as well. The total rate of cell division was little less than in the control. There are two possible explanations for the extended capacity of explants to undergo cell division after addition of 5-FU and thymidine at the end of the first division. The tissue may have become resistant in some way to 5-FU. This is

unlikely, as 5-FU added to explants at 48 h inhibited RNA synthesis as much as 5-FU added at 0 h, and [^{3}H]5-FU was incorporated into RNA at 48 h (Table 2). Furthermore, analysis on sucrose gradients of RNA labelled from 48·5–50·5 h after addition of 5-FU at 48 h showed radioactivity profiles similar to those shown in Fig. 2 for RNA labelled during the first 2·5 h of culture. RNA labelled by [^{3}H]5-FU at 48 h was similar to that labelled from 0–2 h (Fig. 2).

TABLE 2. *Incorporation of [8-^{3}H]adenine and [6-^{3}H]5-FU into RNA in artichoke tuber explants*

Various additions of 5-FU (1 mM) and thymidine (0·33 mM) were made to cultures at 0 or 48 h.

Isotope	Labelling period (h)	Treatment	[^{3}H] radioactivity incorporated into RNA (ct min^{-1} μg^{-1} RNA)
[8-^{3}H]adenine	0·5–2·5	Control	5160
	0·5–2·5	5-FU and thymidine added at 0 h	2580
	48·5–50·5	Control	14 800
	48·5–50·5	5-FU and thymidine added at 48 h	5790
[6-^{3}H]5-FU	0–2	..	348
	48·5–50·5	..	771

The alternative explanation is that the relevant mRNAs and proteins synthesized before addition of 5-FU and thymidine at the end of the first division may have been sufficiently stable to support at least three further divisions in the explant.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council. Some early experiments were carried out in the Department of Botany, University of Edinburgh. I thank U. E. Loening and M. M. Yeoman for advice and helpful discussions.

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Printed in Great Britain
by William Clowes & Sons Limited
London, Colchester and Beccles

Eur. J. Biochem. 50, 529–537 (1975)

Studies on Messenger and Ribosomal RNA Synthesis in Plant Tissue Cultures Induced to Undergo Synchronous Cell Division

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(Received September 30, 1974)

Messenger and ribosomal RNA metabolism was studied in a plant tissue culture system: cells from the quiescent tubers of *Helianthus tuberosus* (Jerusalem artichoke) were induced to divide synchronously and dedifferentiate by excision and culture of explants in nutrient medium. Large accumulations of ribosomal RNA and protein started early in the 20-h lag-period preceding the first division.

In pulse-labelling experiments, two types of polydisperse messenger-like RNAs were detected, one with and one without a poly(adenylic acid) sequence. In the first 2 h of culture the two polydisperse RNA fractions were the predominant types of RNA synthesised. Ribosomal RNA synthesis was very low during the first 2 h, but accelerated later.

Low concentrations of actinomycin-D strongly inhibited ribosomal RNA synthesis, but had little effect on the synthesis of transfer RNA or either type of polydisperse messenger-like RNA. In explants cultured with low concentrations of actinomycin-D ribosomal RNA accumulation was completely inhibited, but cell division and protein accumulation occurred, though at a reduced rate. It is concluded that the synthesis and accumulation of new ribosomal RNA which normally occur during culture are not required for the induction of cell division or for protein accumulation, *i.e.* the ribosomal RNA existing in the quiescent tuber cells can support protein accumulation and cell division induced by excision and culture.

The quiescent tuber tissue is also shown to contain significant amounts of messenger-like RNA.

In nature the storage parenchyma cells of the nature Jerusalem artichoke tuber do not divide, but are quiescent from autumn to spring, when the tuber breaks down to support the growth of the developing buds. However, when explants of the tuber tissue are cultured in nutrient medium, vigorous cell division is induced after a 20-h lag-period [1]. The early cell divisions are inherently highly synchronous [2], the response of the explants is highly consistent from experiment to experiment and almost all the cells of the explants divide [3]. Repeated cell division, in the virtual absence of cell expansion, leads to a tissue of small, meristematic-type cells [1]. Before and during the early divisions the rate of rRNA synthesis increases, and large accumulations of rRNA and protein occur. These changes are not continuous but, like cell division, occur in a stepwise or synchronous manner [4]. This culture system provides an opportunity to study the role of RNA synthesis in a situation similar to oncogenesis, where non-dividing specialised cells are induced to divide and dedifferentiate. This paper reports studies on mRNA and rRNA metabolism during the early part of culture.

It is highly likely that mRNA metabolism plays a significant part in the control of activation of the tissue following excision, and in the developmental transition of cells from the non-dividing, differentiated state to the dividing, dedifferentiated state. The quiescent tuber tissue is shown to contain appreciable amounts of messenger-like RNA. In the period of culture immediately after excision, messenger-like RNA was found to be the predominant species synthesised. Two types of polydisperse, messenger-

Abbreviations. Poly(A), poly(adenylic acid); poly(A)$^+$-RNA, RNA containing a poly(adenylic acid) sequence; poly(A)$^-$RNA, RNA not containing a poly(adenylic acid) sequence; poly(U), poly(uridylic acid); oligo(dT), oligo(thymidylic acid).

like RNA are described, one with and one without a poly(A) sequence.

The quiescent tuber cells also contained rRNA [4]. I investigated whether this existing rRNA could support the accumulation of protein and cell division, or whether the large amounts of new rRNA synthesised during early culture [4] were required for the acceleration of protein synthesis and the induction of cell division. These possibilities were tested using the DNA-binding antibiotic actinomycin-D. Low concentrations of actinomycin-D have been shown selectively to inhibit rRNA synthesis in mammalian cells, while not inhibiting mRNA synthesis [5–7]. This paper shows a similar selective effect of low concentrations of actinomycin-D in plant cells.

MATERIALS AND METHODS

Tissue Culture

The primary explants of Jerusalem artichoke (*Helianthus tuberosus* L. var. Bunyard's Round) tuber tissue were 9 mg fresh weight, 2.5 mm long and 2 mm diameter, and contained 20000 cells of which 99% were secondary xylem parenchyma. The liquid culture medium contained 4% sucrose, salts [8] and 10 μM 2,4-dichlorophenoxyacetic acid. Explants were cultured in groups of 30, in 5.25 ml medium, in a 90-mm diameter petri dish, in total darkness. Cultures were agitated on a reciprocating shaker with a 50-mm displacement operating at 50 cycles/min (0.83 Hz). Culture was carried out under fully sterile conditions. No bacterial growth was detectable when samples of cultures were plated out on nutrient agar. Other details of the culture system have been published previously [1, 3, 4].

Percentage increase in cell number was estimated from samples of 5 explants macerated for 24 h in 2 ml 5% chronic acid. The method used to estimate cell number was based on the finding that the cell walls of the original cells of the explant do not degrade during maceration. Thus the first division leads to the appearance of cell pairs, *i.e.* two daughter cells within the parent cell wall; similarly cell fours appear in the macerate when an original cell has divided twice. Details of how percentage increases in cell-number data were derived from the macerates, and experimental and statistical verification of the reliability of the counting technique are published elsewhere [4].

It was possible by this method to recognise unequivocally the timings of the first and second divisions, and to determine how an increase in cell number had occurred. For example, it was possible to distinguish between an increase caused by a large number of cells dividing once, or a small proportion of cells dividing repeatedly.

Total RNA content was measured from the ultraviolet absorption spectrum of extracted RNA [9] after washing the explants [10] to remove ultraviolet-absorbing contaminants. The ultraviolet absorption spectra showed little sign of contamination, with 260:235 nm absorption ratios of over 2:1.

Protein content was measured by the method of Lowry *et al.* [11]. Each total RNA or protein determination was performed on a sample of 10 explants. Experimental points quoted in this paper are means of 3 to 8 determinations on samples from replicate cultures. Fuller details of the methods of estimating total RNA and protein contents are published elsewhere [4].

Actinomycin-D was dissolved in water at 10–50 times the required final concentration and added aseptically to cultures through a 0.22-μm Millipore filter.

Uptake and Incorporation of [5-^{3}H]Uridine

Explants were incubated with 20 μCi/ml [5-^{3}H]-uridine (Radiochemical Centre, Amersham, U.K.; specific activity 25 Ci/mmol) for 2-h periods. Groups of ten explants were lightly blotted and their fresh weight was measured.

For isotope uptake measurement explants were rinsed with ice-cold culture medium, then extracted with 1 ml 0.5 N perchloric acid at 70 °C for 30 min. 10-μl samples of the extract were dried on 15-mm squares of Whatman GF/A glass-fibre paper and counted in 5 ml 0.5% (w/v) 2-(4′-tert-butylphenyl-5-(4″-biphenylyl)-1,3,4-oxadiazole (butyl-PBD)–toluene scintillator.

For measurement of the incorporation of isotope into RNA, explants were washed in chilled culture medium and homogenised at 0 °C in 5 ml 5% trichloroacetic acid, using a motor-driven teflon-in-glass homogeniser. The precipitate was collected by filtration on Whatman GF/A paper, washed three times with 5% trichloroacetic acid and twice with 80% (v/v) ethanol containing 0.1 M NaCl, dried and counted as above.

Uptake and incorporation of uridine were expressed per mg fresh weight of tissue.

Labelling and Extraction of RNA

Explants were incubated with 100 μCi/ml [5-^{3}H]-uridine for 2-h periods. Groups of 50 explants were homogenised in 5 ml 2% (w/v) sodium triisopropylnaphthalene sulphonate, 50 mM Tris-HCl pH 7.8, 10 mM NaCl, at 20 °C, in a motor-driven teflon-in-

glass homogeniser. The homogenate was emulsified with an equal volume of phenol containing 20% (v/v) *m*-cresol [12], 20% (v/v) chloroform and 0.5% (w/v) 8-hydroxyquinoline for 5 min, then centrifuged for 5 min at $2000 \times g$. The aqueous phase was further deproteinised, nucleic acids were precipitated by ethanol and then purified by successive reprecipitations as described previously [13].

The total RNA content of the extract was estimated from the ultraviolet absorption spectrum of a sample hydrolysed in 0.5 N perchloric acid at 70 °C for 20 min. The value of absorption at 260 nm was converted to a figure for μg RNA/ml using a molar absorption value of $12400\ M^{-1} \times cm^{-1}$, calculated for a solution of equal molar quantities of the four ribonucleotides at acid pH.

Isolation of Poly(A)-Containing RNA

100–150 μg of total RNA was dissolved in 1 ml binding buffer (400 mM NaCl, 10 mM Tris-HCl pH 7.8, 1 mM EDTA, 0.2% sodium dodecylsulphate) and shaken gently with 20 mg oligo(dT)-cellulose [14, 15] [Collaborative Research Inc., Waltham, Mass., U.S.A.; binding capacity 42.8 A_{260} units of poly(A)/g] for 30 min at 20 °C. The cellulose was sedimented at $12000 \times g$ for 2 min, then washed four times with 0.5-ml aliquots of binding buffer. The supernatants were combined and the "poly(A)$^-$RNA" fraction precipitated from them with 2.5 volumes of ethanol.

The cellulose was then washed four times with 0.5 ml elution buffer (binding buffer *minus* NaCl) at 20 °C. The "poly(A)$^+$RNA" fraction was precipitated with alcohol from the combined supernatants after addition of 50 μg non-radioactive artichoke carrier RNA and NaCl to 0.3 M.

Sucrose Density Gradient Fractionation of RNA

50-μg samples of RNA, dissolved in 100 μl gradient buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.8, 1 mM EDTA, 0.5% sodium dodecylsulphate) were layered on to 5-ml gradients of 5–25% (w/v) sucrose in gradient buffer. The gradients were centrifuged for 2.5 h at 50000 rev./min (g_{av} 234000) in a Spinco SW 50.1 rotor, at 20 °C or 25 °C.

Absorbance at 265 nm was monitored by displacing the gradients by heavy sucrose solution through a flow cell in a Joye-Loebl scanner. 36 12-drop fractions were collected. RNA was hydrolysed by adding 0.2 ml 0.5 N perchloric acid to each fraction and incubating at 70 °C for 20 min. Fractions were then mixed with 9 ml scintillator, *i.e.* 0.5% (w/v) 2-(4′-tert-butylphenyl-5-(4″-biphenylyl)-1,3,4-oxadiazole; 40% (v/v) 2-methoxyethanol; 60% (v/v) toluene and counted.

Detection and Assay of Poly(A)-Containing RNA

Non-radioactive RNAs containing poly(A) sequences were detected and assayed by hybridisation with [^{3}H]poly(U) followed by sucrose density gradient centrifugation, as described elsewhere [16]. [^{3}H]-poly(U) was purchased from Miles Laboratories.

RESULTS

Synthesis of Polydisperse RNA and rRNA during the Early Hours of Culture

Poly(adenylic acid) sequences have been found in the mRNAs of a wide variety of eukaryotes, including mammals [15, 17–19], yeasts [20, 21] and higher plants [22, 23]. Methods have been developed for the isolation of poly(A)-containing RNA from total RNA, based on hybridisation of the poly(A) region to immobilised complementary homopolynucleotide. Radioactive artichoke RNA was separated into poly(A)-containing RNA [poly(A)$^+$RNA] and non-poly(A)-containing RNA [poly(A)$^-$RNA] using the selective binding of poly(A)$^+$RNA to oligo(dT)-cellulose at high salt concentration [14, 15].

Hepburn (unpublished results) has shown that the fraction of artichoke RNA which binds to oligo(dT)-cellulose at high salt concentration contains a polynucleotide sequence with very high A content, resistant to degradation by pancreatic or T_1 RNase, and with a length of about 150 nucleotide residues. The RNA which does not bind to oligo(dT)-cellulose does not contain such an A-rich sequence.

The poly(A)$^+$ and poly(A)$^-$RNA fractions prepared from total artichoke radioactive RNA are shown in Fig. 1 and 2. In explants incubated with [5-^{3}H]uridine for the first 2 h of culture the radioactive poly(A)$^+$RNA had a polydisperse distribution when fractionated on a sucrose gradient (Fig. 1B). The mean sedimentation coefficient was about 18 S, the range was from 6 S to over 30 S. The poly(A)$^+$-RNA synthesised from 3–5 h had a similar lower limit of size, but was on average slightly smaller, with a mean size of 16 S (Fig. 2B).

The poly(A)$^-$RNA synthesised in explants from 3 to 5 h is shown in Fig. 2A. There is clear labelling of 18-S and 25-S rRNAs and tRNA, but also a high background of polydisperse labelled RNA, ranging in size from about 7 S to over 30 S. In tissue labelled from 0–2 h, rRNA synthesis was much less marked, and the predominant forms of poly(A)$^-$RNA synthesized were tRNA and the 7–30-S polydisperse fraction.

Control experiments showed that the polydisperse RNA in the poly(A)$^-$RNA fraction was not poly(A)$^+$-RNA which had failed to bind to the oligo(dT)-

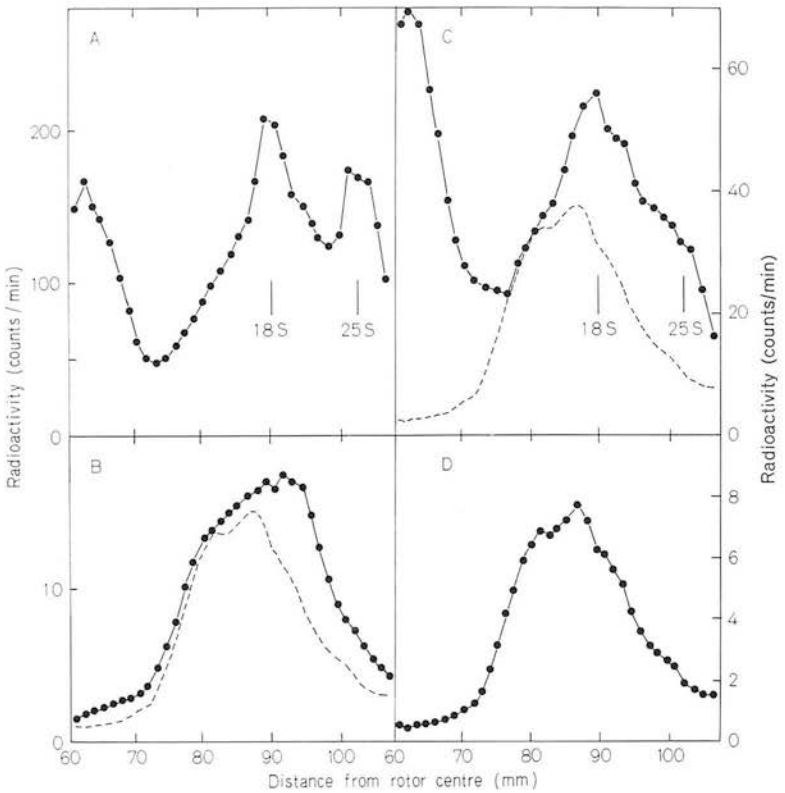

Fig. 1. *Sucrose density gradient sedimentation of artichoke tuber RNA labelled from 0–2 h of culture.* (●——●) [^{3}H]-Radioactivity; all values were corrected for the amount of total RNA applied to the oligo(dT)-cellulose, and are expressed per 100 µg total RNA. (A) Poly(A)$^-$RNA and (B) poly(A)$^+$RNA from control explants. (C) poly(A)$^-$RNA and (D) poly(A)$^+$RNA from explants treated with 25 µg/ml actinomycin-D. The broken lines in (B) and (C) show the profile of radioactivity in (D) redrawn to permit size comparisons. The positions of the 25-S and 18-S ribosomal RNA ultraviolet absorbance peaks are shown

cellulose. When the poly(A)$^-$RNA fraction was reprocessed with a fresh batch of oligo(dT)-cellulose, the radioactivity binding to the cellulose at high salt concentrations was only 10–15% of the radioactivity of the original poly(A)$^+$RNA fraction. Conversely, the polydisperse poly(A)$^+$RNA fraction did not appear to be significantly contaminated by poly(A)$^-$-RNA. When poly(A)$^+$ fractions were reprocessed with fresh oligo(dT)-cellulose, 95–100% of the radioactivity again bound to the cellulose at high salt concentration. These results confirm that oligo(dT)-cellulose separated artichoke RNA into poly(A)$^+$ and poly(A)$^-$ fractions, and show that there was little or no contamination of one fraction by the other. Finally, the total radioactivity recovered from the cellulose was about 95% of the radioactivity applied.

Table 1. *Effects of various concentrations of actinomycin-D, added at the start of culture, on uptake and incorporation of [5-^{3}H]uridine by artichoke tuber explants during 2-h pulse incubations started after 0 or 3 h of culture*

Data are expressed as percentages of values in control explants grown without actinomycin-D. Absolute control values were (counts × min^{-1} × mg fresh weight^{-1}): 0–2 h, uptake 1570, incorporation 80; 3–5 h, uptake 2200, incorporation 387

Time of pulse incubation	Experiment	Actinomycin-D (µg/ml)		
		5	25	50
h		% of control		
0–2	uridine uptake	157	122	106
	incorporation	76	33	31
3–5	uridine uptake	110	–	33
	incorporation	40	–	3

Actinomycin-D and RNA Synthesis

Table 1 shows that actinomycin-D inhibited RNA synthesis in cultured explants. The extent of inhibition increased with increasing actinomycin-D concentration and with increased time of exposure to the drug. The inhibition of [5-^{3}H]uridine incorporation ob-

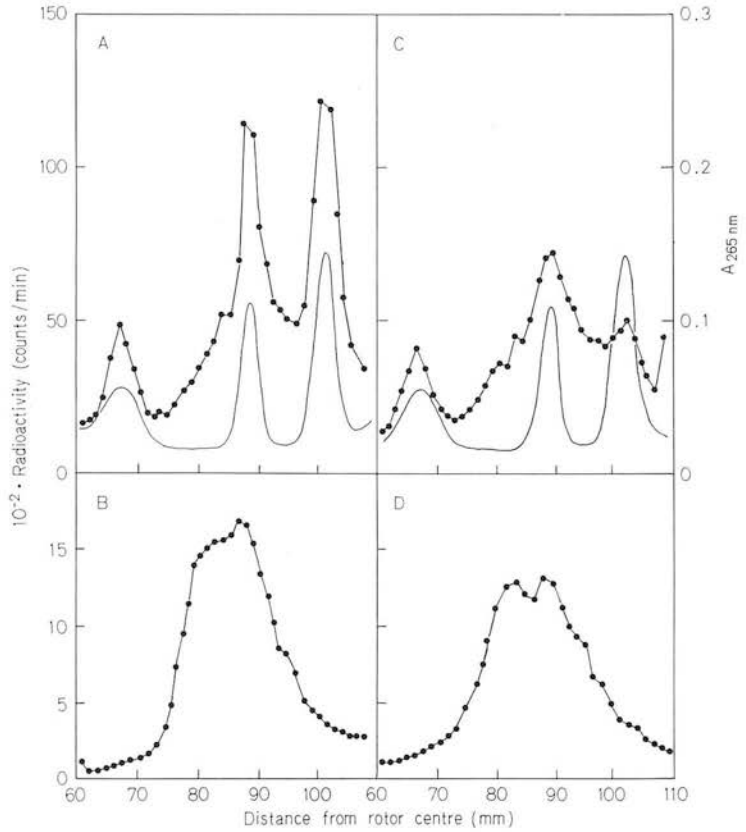

Fig. 2. *Sucrose density gradient sedimentation of artichoke tuber explant RNA labelled from the 3rd to 5th h of culture.* (●——●) [^{3}H]Radioactivity, expressed per 100 µg total RNA fractionated on oligo(dT)-cellulose. Continuous line: absorbance at 265 nm. (A) Poly(A)$^-$RNA and (B) poly(A)$^+$-RNA from control explants; (C) poly(A)$^-$RNA and (D) poly(A)$^+$RNA from explants treated with 5 µg/ml actinomycin-D from the start of culture

served was a result of a genuine inhibition of RNA synthesis rather than an inhibition of isotope uptake: low concentrations of actinomycin-D, or high concentrations for short periods, actually stimulated the uptake of uridine. Very similar effects of actinomycin-D on RNA synthesis were found using [8-^{3}H]-guanosine as label, but actinomycin did not significantly stimulate the uptake of guanosine.

Fig. 2 shows the effects of 5 µg/ml actinomycin-D on the synthesis of particular types of RNA between 3 and 5 h after the start of culture. The synthesis of poly(A)$^+$RNA was very little inhibited by actinomycin-D (Fig. 2D). Of the poly(A)$^-$RNAs (Fig. 2C), the syntheses of tRNA and the polydisperse RNA were not inhibited, but the synthesis of rRNA was strongly inhibited. The radioactivity of the 25-S rRNA in actinomycin-D-treated explants was only 20% of that in the control (Fig. 2A). 18-S rRNA radioactivity was also very reduced by actinomycin-D, but it is less easy to compare the 18-S radioactivities because of the high background of polydisperse radioactivity which has a mean around 18 S.

When tissue was exposed to a higher level of actinomycin-D (25 µg/ml) and labelled from 0–2 h, the synthesis of rRNA was very strongly inhibited (Fig. 1C). In the poly(A)$^-$ fraction only tRNA and polydisperse RNA were labelled, though the synthesis of both was inhibited by about 50% (Fig. 1C). The synthesis of poly(A)$^+$RNA was also inhibited by about 50% (Fig. 1D). The poly(A)$^+$RNA labelled in the presence of 25 µg/ml actinomycin-D (Fig. 1D) was significantly smaller in mean size than that labelled in control cells (Fig. 1B), though the lower limit of the size range was the same for both.

The concentration of actinomycin-D required to inhibit RNA synthesis in artichoke tissue was similar to those reported effective in other plant tissues [24], but much higher than the concentrations required to inhibit RNA synthesis in mammalian cells [5–7]. It is clear than at low actinomycin-D concentrations, or in short exposures to high concentrations, rRNA synthesis in artichoke tissue was very strongly inhibited, while the synthesis of tRNA and both polydisperse messenger-like fractions was very much less

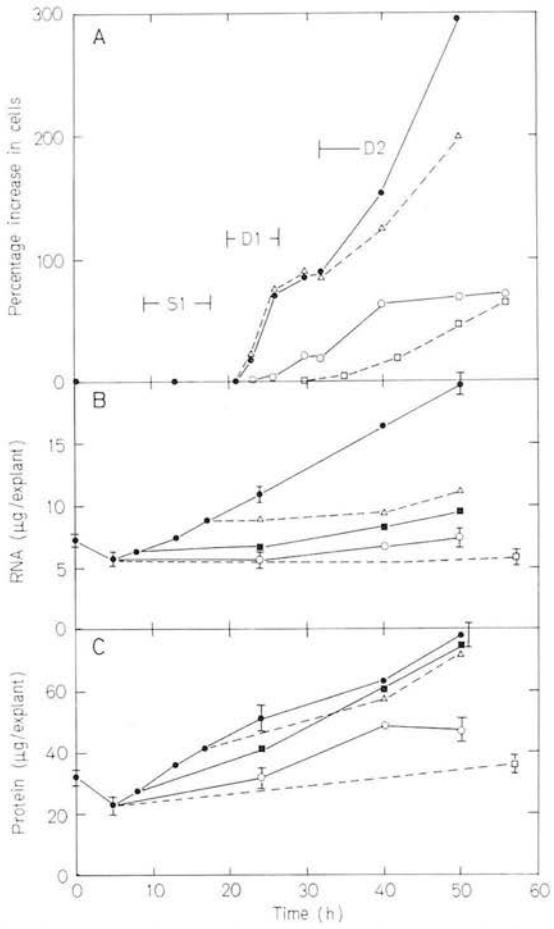

Fig. 3. *Cell division, RNA and protein accumulation during culture of artichoke tuber explants.* (A) Percentage increase in cell number. D1 and D2 show the timings of the first and second cell divisions; S1 the timing of DNA synthesis for the first division. These timings were established as explained in Materials and Methods and [4]. (B) µg RNA per explant. (C) µg protein per explant. (●——●) Control; (○——○) 5 µg/ml actinomycin-D present from 0 h; (■——■) 5 µg/ml actinomycin-D added at 8 h; (△- - -△) 5 µg/ml actinomycin-D added at 18 h; (□- - -□) 20 µg/ml actinomycin-D added at 0 h. Each RNA and protein value quoted is the mean of between three and eight replicate determinations. The vertical bars indicate standard deviations of those points which are the means of eight determinations

inhibited. This type of selective inhibition of rRNA synthesis by lower concentrations of actinomycin-D is well known in animal cells [5–7] but does not appear to have been reported previously for plants.

Actinomycin-D and Cell Division

The object of this part of the investigation was to discover if the large increase in total RNA content of explants which starts long before the first division (shown in Fig. 3, and described in detail in [4]) is necessary for the occurrence of cell division and the accompanying increase in explant protein content. Explants were grown with low concentrations of actinomycin-D to inhibit selectively the synthesis of rRNA, which is about 80% of total RNA.

Fig. 3 shows that when 5 µg/ml actinomycin-D was present from the start of culture, cell division was delayed, then occurred at a reduced rate. Examination of the chromic acid macerate of 57-h explants for products of the first and second divisions, as explained in Materials and Methods, showed that the increase in cell number was entirely due to cells undergoing the first division. By 57 h no cells had undergone a second division, but almost as many cells had completed the first division as did so, earlier, in control explants.

In contrast, the accumulation of RNA was almost totally inhibited by 5 µg/ml actinomycin-D; certainly no net increase in RNA content occurred until cell division was well under way. 20 µg/ml actinomycin-D gave complete inhibition of RNA accumulation; cell division occurred, though at a much slower rate than in the control.

When actinomycin-D was added to cultures at 18 h, just before the first division, RNA accumulation ceased immediately. The first division was completely uninhibited, but the second division was rather slower than in the control.

These results, together with the known effects of actinomycin-D on rRNA synthesis, show that the massive synthesis and accumulation of new ribosomal RNA which occur in control explants (Fig. 2, 3) [4], are not required for the induction of the first cell division.

Actinomycin-D and Protein Accumulation

Explants in which rRNA accumulation had been totally inhibited by actinomycin-D added at the start of culture did accumulate protein, though more slowly than in the control (Fig. 3). This shows that the massive synthesis and accumulation of new rRNA occurring in control explants was not necessary for protein accumulation. When actinomycin-D was added to 5 µg/ml at the start of culture the rate of protein accumulation was about half that in the control, and accumulation ceased after 40 h. Fig. 2 shows that this concentration of actinomycin-D had little inhibitory effect on the synthesis of messenger-like RNA 3–5 h after the start of culture, but the amount of inhibition did increase later, presumably as the drug increased in concentration within the cells. There is also the possibility that although mRNA synthesis was initially comparatively uninhibited, protein synthesis was limited by an actinomycin-D-sensitive RNA species required for translation of

mRNA [25]. 20 μg/ml actinomycin-D added at the start of culture severely reduced protein synthesis. This is consistent with the demonstrated inhibition of mRNA synthesis by high concentrations of the antibiotic (Fig. 1).

When 5 μg/ml actinomycin-D was added to cultures at 8 h, protein accumulation continued almost as rapidly as in control explants until at least 50 h, although net RNA accumulation ceased immediately (Fig. 3). Comparing the effects on protein synthesis of 5 μg/ml actinomycin-D added at 0 h and 8 h, it would seem that in the first 8 h of culture the explants aquire the ability to synthesise proteins for extended periods despite the presence of actinomycin-D. One possible explanation of this could be the synthesis of relatively long-lived messengers early in the culture period.

The initial loss of RNA and protein from explants during the first hours of culture (Fig. 3) was loss of material from cells on the outside of the explant, which were broken or damaged during excision.

Detection of mRNA in Freshly-Excised Explants

Mature artichoke tubers are harvested in autumn and stored at 4 °C for up to 12 months. During storage they are assumed to be metabolically sluggish. Changes so far detected are that after 6 months' storage the initial protein and RNA contents of explants begin to decline [26], and there is an increase in the duration of the lag-period before the first division in culture [2, 4]. It was of interest to see if the quiescent tuber contained mRNA.

Total RNA from tubers stored for 5 months after harvesting was fractionated on a sucrose density gradient after mixing with [^{3}H]poly(U) [16]. A sample of [^{3}H]poly(U) sedimented alone had a mean sedimentation coefficient of 3 S (Fig. 4), and no significant amount of radioactivity sedimented to heavier regions of the gradient. When mixed with artichoke RNA some poly(U) radioactivity sedimented to the heavier regions of the gradient, to give a polydisperse distribution of radioactivity with a mean of over 18 S. This poly(U) is taken to have hybridised to RNAs having poly(A) sequences. The distribution of radioactivity therefore shows the distribution of poly(A)$^+$RNAs.

In confirmation of this interpretation, when non-radioactive artichoke RNA was sedimented alone, then each fraction of the gradient was challenged with [^{3}H]poly(U), the distribution of radioactivity remaining precipitable by trichloroacetic acid after RNase treatment [27] was similar to the 6–30-S polydisperse distribution show in Fig. 4. This indicates that the poly(U) was hybridised to poly(A) sequences,

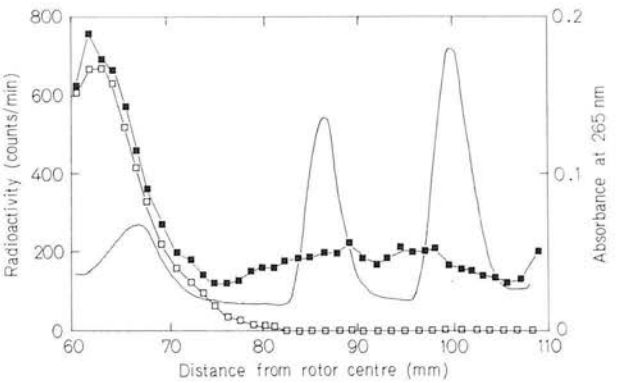

Fig. 4. *Sucrose gradient sedimentation of mixtures of non-radioactive artichoke tuber RNA and [^{3}H]poly(U).* Continuous line: absorbance at 265 nm. (□——□) Radioactivity profile of 25 ng [^{3}H]poly(U) sedimented alone. (■——■) Radioactivity profile of 0.05 μg [^{3}H]poly(U) sedimented with 50 μg non-radioactive artichoke tuber RNA prepared from freshly cut tuber tissue

and that the radioactivity shown sedimenting to heavy regions of the gradient in Fig. 4 was not carried there by non-specific aggregation with poly(A)$^-$RNA, or by binding to some contaminant in the RNA.

It is possible to estimate the amount of poly(A)$^+$-RNA present in total artichoke tuber RNA from the data shown in Fig. 4. 0.05 μg [^{3}H]poly(U) was mixed with 50 μg total RNA; about one-half of the poly(U) hybridised and was carried down the gradient. Assuming that this poly(U) was hybridised to an equal weight of poly(A) [27], the poly(A) content of the total RNA was 0.025 μg per 50 μg, *i.e.* 0.05%. Knowing the average length of poly(A) sequences in artichoke tuber tissue RNA to be 150 nucleotides (A. Hepburn, unpublished results) and the average size of the poly(A)$^+$RNA (in Fig. 4 about 2000 nucleotides), the poly(A)$^+$RNA content of the total RNA can be calculated as 0.67%. Because of the assumptions made, this is a very approximate value but it does indicate that the quiescent tuber tissue contains appreciable amounts of messenger-like RNA. For comparison, the much smaller 9-S globin mRNA is about 1% of total RNA in mouse reticulocytes [16].

DISCUSSION

This study has shown the presence of two RNA fractions in artichoke tuber tissue which have some characteristics of messenger RNA. They are heterogeneous in weight; the actual size range corresponds well to the sizes of mRNAs which would code for a reasonable distribution of protein sizes. Both fractions were labelled to a high specific activity in a pulse incubation, though it was not possible to calculate the exact specific activity. The weight of

poly(A)$^+$RNA prepared by oligo(dT)-cellulose was too low to measure accurately, and non-radioactive carrier RNA had to be added to ensure complete precipitation of the radioactive poly(A)$^+$RNA. The weight of polydisperse poly(A)$^-$RNA could not be determined as no method exists to separate it from the rRNA and tRNA also present in the poly(A)$^-$ fraction. However, it is clear from Fig. 1 and 2 that both polydisperse poly(A)$^+$ and poly(A)$^-$RNAs were labelled to a very much higher specific activity that rRNA or tRNA.

The synthesis of both polydisperse fractions was relatively resistant to inhibition by actinomycin-D, as are mammalian messenger and nuclear heterogeneous RNA syntheses [5–7]. One of the fractions contains a poly(A) sequence, the other does not. There are two further differences between the polydisperse poly(A)$^+$ and poly(A)$^-$RNAs. First, labelling of the poly(A)$^-$ polydisperse was about ten times that of the poly(A)$^+$ fraction (Fig. 1, 2). Secondly, the polydisperse poly(A)$^-$RNA appears to be heavier than the poly(A)$^+$RNA. This point is shown most clearly where rRNA synthesis was inhibited by actinomycin-D (Fig. 1C, D) but appears also to be true in control tissue RNA. However, to establish unequivocally that the difference in mean sedimentation coefficient was due to a difference in molecular weight would require fractionation under denaturing conditions, such as on formamide–polyacrylamide gels.

These two fractions of polydisperse RNA bear close resemblance to two fractions of soybean RNA isolated by methylated albumin–kieselguhr chromatography by Key *et al.* [28]. They described "TB-RNA", a light fraction with high adenylic acid content, and "D-RNA", a heavier polydisperse fraction with lower adenylic acid content.

A. Hepburn (unpublished results) has shown in artichoke tissue that poly(A)$^+$RNA is associated with the polyribosomes. It is thus likely that the poly(A)$^+$-RNA described here is at least partly cytoplasmic mRNA. The role of the polydisperse poly(A)$^-$RNA remains unclear, though cytoplasmic mRNAs without poly(A) sequences do exist in eukaryotes [20, 23, 29]. The possibility cannot be entirely excluded that the polydisperse poly(A)$^-$RNA was partly poly(A)$^+$RNA which had lost its poly(A) sequence during extraction. However, the apparently larger size of the poly(A)$^-$ polydisperse fraction, and the consistently large amounts labelled during a pulse incubation suggest similarity to the poly(A)$^-$ fraction of nuclear heterogeneous RNA in mammalian cells [30]. Further study of the kinetics of labelling of these two polydisperse fractions and of their intracellular location should be of interest.

The synthesis of messenger-like RNA was an especially prevalent feature of total RNA synthesis during the early part of explant growth. The poly(A)$^+$-RNA alone, measured as the percentage of radioactivity binding to the oligo(dT)-cellulose, was 7.2% of the total RNA synthesised during 0–2 h, and fell to 2.7% during the period 3–5 h. This did not reflect a drop in the absolute rate of poly(A)$^+$RNA synthesis, but was a consequence of the comparatively late acceleration of rRNA synthesis (Fig. 1A, 2A). Verma and Marcus [31] found that during transition from stationary phase to active growth in cultures of *Arachis* cotyledon cells, messenger-like RNA synthesis was at first predominant, with rRNA synthesis occurring later. Similarly, when spores of fission yeast germinate, there is a rapid early rise in mRNA content, followed later by accelerating rRNA synthesis [21].

The quiescent artichoke tuber tissue contained a significant amount of poly(A)-containing, messenger-like RNA (Fig. 4). Ungerminated fission yeast spores also contain poly(A)$^+$RNA [21], and preformed messenger-like RNA has been detected in quiescent wheat embryos [32, 33]. It will be interesting to see if the stored messenger-like RNA of the artichoke tuber tissue is translated during culture, or if the completely new developmental pathway induced by excision and culture renders this messenger useless. The poly(A)$^+$RNA stored in tuber tissue (Fig. 4) appeared to be larger than the poly(A)$^+$RNA synthesised during the early part of culture (Fig. 1, 2).

The experiments on longer-term cultures with actinomycin-D (Fig. 3) showed that the massive synthesis and accumulation of rRNA occurring before division in control explants [4] were not required to support the induction of cell division or the occurrence of protein accumulation. These data, however, do not exclude the possibility that the very small amount of rRNA synthesis occurring early after addition of actinomycin-D (Fig. 1, 2) might not be of importance for the induction of cell division and protein synthesis. They do show that no significant increase in the size of the ribosomal population is required.

In a previous paper [4], it was shown that the normal stepwise accumulation of rRNA during culture does not depend for its occurrence or timing on the occurrence of DNA synthesis and cell division: when DNA synthesis was completely inhibited by mitomycin-C, rRNA was accumulated in a stepwise manner as normally. These two results strongly suggest that these is not a close coordination, in the short term at least, between the synchronous cell division growth pattern induced in the tissue and the synthesis or accumulation of rRNA.

In work with expanding soybean cells it has been shown [34, 35] that the continuance of cell expansion does not depend on continued rRNA synthesis. The essential difference between these results and the artichoke system is that in artichoke explants it is the induction of a completely new developmental process in quiescent tissue, which does not require rRNA synthesis, rather than the continuance of an established development as in soybean.

In Fig. 3 the considerable delay to cell division, and strong inhibition of protein accumulation by 20 μg/ml actinomycin-D might be taken to imply a requirement for mRNA synthesis for the occurrence of cell division and protein accumulation. However, such data must be interpreted with caution, because of possible indirect inhibition by actinomycin-D of mRNA translation [25] and direct inhibition of DNA synthesis by high concentrations of actinomycin-D [36]. A separate paper will describe experiments with 5-fluorouracil, which in the presence of thymidine affects RNA synthesis but not DNA synthesis. Application of 5-fluorouracil after various times of culture has allowed the identification of two periods of mRNA synthesis necessary for the occurrence of the first and subsequent divisions.

This work was supported by the Medical Research Council. Some preliminary experiments were carried out in the Department of Botany, University of Edinburgh. I thank Angus Hepburn for allowing me to quote his data prior to publication, Chris Leaver and Ulrich Loening for discussions, and Mike Yeoman for a supply of artichoke tubers.

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Planta 148, 417–421 (1980)

Planta

Ribosomal RNA Integrity and Rate of Seed Germination

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Abstract. The integrity of ribosomal RNA (the percentage of complete, un-nicked molecules) in seeds was studied by electrophoresis under denaturing conditions. Two batches of carrot seed, harvested at different stages of maturity, and four batches of *Nicotiana* seed stored for various times were used. Within each species, there was a correlation between the integrity of the rRNA of the dry seed and the rate of germination of that seed. In carrot seed, there was extensive degradation of existing rRNA in both the embryo and endosperm during the first two days of imbibition.

Key words: *Daucus* – Germination (seeds) – *Nicotiana* – rRNA – Seed ageing – Seed germination.

Introduction

Successful seed germination and seedling growth depend on protein synthesis (Brooker et al. 1977); this in turn requires that seeds have an adequate supply of functional ribosomes. Previous reports have shown that loss of viability is accompanied by degradation of the rRNA of the dry seed (Roberts et al. 1973; Bray and Chow 1976).

For many horticultural crops, it is also desirable that seeds have rapid and uniform germination. We are therefore interested in possible biochemical explanations of why different batches of seed of similar, high levels of viability can germinate at very different rates. We report here that seed of high viability can show extensive degradation of rRNA. There is a correlation between rate of germination of different batches of seed and the integrity of rRNA, both in dry seed and during the course of germination.

Abbreviations: rRNA = ribosomal RNA; tRNA = transfer RNA

Materials and Methods

Seeds

Carrot (*Daucus carota* L., cv. Royal Chantenay) plants were grown in a glasshouse in 1974. Seed was collected from the primary umbels, 44 and 104 days after anthesis (immature and mature seed, respectively), air dried and stored at 7–10° C, 50% relative humidity. Plants of *Nicotiana tabacum* L., cv. White Burley and *Nicotiana glutinosa* L. were grown in a glasshouse in 1973 and 1978. Seed was air dried and stored in air-tight bottles at 4° C. The experiments were done in 1979.

Germination Tests

Fifty seeds of each batch were placed on two layers of filter paper in 90 mm petri dishes and moistened with 3 ml distilled water. There were eight replicates per batch. All seeds were germinated in the light, carrot at $10 \pm 1°$ C, *Nicotiana* at $22 \pm 1°$ C. The lower temperature was chosen for carrot to give a clear separation between the germination times of the two seed batches. The number of seeds germinated (having a visible radicle) was recorded daily until no further germination occurred. From these data the final percentage germination and the mean germination time were calculated (Orchard 1977).

Extraction of Nucleic Acids

One hundred mg of dry tobacco seeds, or samples of 200 seeds of carrot at various stages of germination, were ground in a pestle and mortar in 4 ml extraction medium (2% sodium tri-isopropylnaphthalene sulphonate; 10 mM NaCl; 50 mM Tris-HCl pH 7.8; 0.2% 2-mercaptoethanol; 0.2% silicone DC antifoam emulsion (Dow Corning)). Homogenization was completed in a motor-driven teflon-in-glass homogenizer. Protein was removed by successive phenol extractions and nucleic acids further purified by reprecipitations with ethanol from 0.5% sodium dodecyl sulphate in 150 mM pH6 sodium acetate and dialysis against the same solution (Fraser and Whenham 1978). Total yield of nucleic acid was measured from its ultraviolet absorption spectrum.

Other batches of immature carrot seeds were dissected into embryo and 'endosperm' (endosperm plus seed coat) fractions; nucleic acids were extracted from 50 embryos and 50 endosperms in the same way as for the whole seeds, except that 2 ml extraction medium was used.

Polyacrylamide Gel Electrophoresis

Samples of 10 to 15 µg total nucleic acid were fractionated on 6 mm diameter polyacrylamide gels (Loening 1969) with final acryl-

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amide concentration equivalent to 2.5%. Electrophoresis was for 3 h at 5 mA per gel, 8 V per cm constant voltage. Gels were washed in distilled water for 2 h, then scanned at 265 nm in a Joyce-Loebl Gel Scanner.

To expose hidden breaks in the rRNA, parallel samples of nucleic acid were fractionated by electrophoresis under denaturing conditions using 99% formamide (Staynov et al. 1972). The gels contained the equivalent of 4% acrylamide and 0.4% N,N'methylene bisacrylamide. Electrophoresis was for 12 h at 1.25 mA per gel, constant current. After electrophoresis, the gels were washed for 5 h in distilled water to remove formamide before scanning at 265 nm.

Weights of rRNA and DNA in gel peaks were calculated from the peak areas, which are linearly proportional to the amount of nucleic acid in the peak (Fraser 1971). The system was calibrated by gel electrophoresis of known amounts of highly purified tobacco mosaic virus RNA and tobacco plant total DNA. The total rRNA content (intact plus degraded molecules) of a nucleic acid sample was calculated by subtracting DNA (measured on gels) and an assumed 20% of the total for tRNA from the measurement of total nucleic acid content of the sample.

Results

Carrot Seed

Both batches of seed had high viability but the mature seed germinated earlier than the immature seed (Table 1). Although the immature seed was harvested only 44 days after anthesis, it had accumulated 90% of the dry weight of the mature seed. The total nucleic acid content of the immature seed was considerably less than that of mature seed. Both batches of seed showed large increases in total nucleic acid over the first 12 days of imbibition.

Figure 1 shows aqueous (non-denaturing) gels of nucleic acids from dry and germinating seeds of the two batches. The same amount of total nucleic acid was applied to each gel. All gels showed peaks of the larger (25 *S*) and smaller (18 *S*) rRNA. Each peak had a shoulder on the lighter side, and there were other small peaks, suggesting partial degradation of the rRNAs. The shoulders and minor peaks were more pronounced in germinating seeds of both batches than in dry seed. A large, disperse region of absorption at 50–75 mm was found in samples with comparatively small 25 *S* and 18 *S* peaks. This highly mobile material probably represented extensively degraded rRNA, and was most common in dry, immature seeds.

Table 1. Characteristics of two batches of carrot seed germinated at 10° C

	Immature seed	Mature seed	L.S.D. ($P \leq 0.05$)
Percentage germination	82	86	4.4
Mean germination time (days)	12.8	9.1	0.37
Dry weight per seed (mg)	1.33	1.47	0.07
Nucleic acid per seed (μg)			
dry seed	1.18	1.68	0.32
after 12 days imbibition	3.0	5.2	1.0

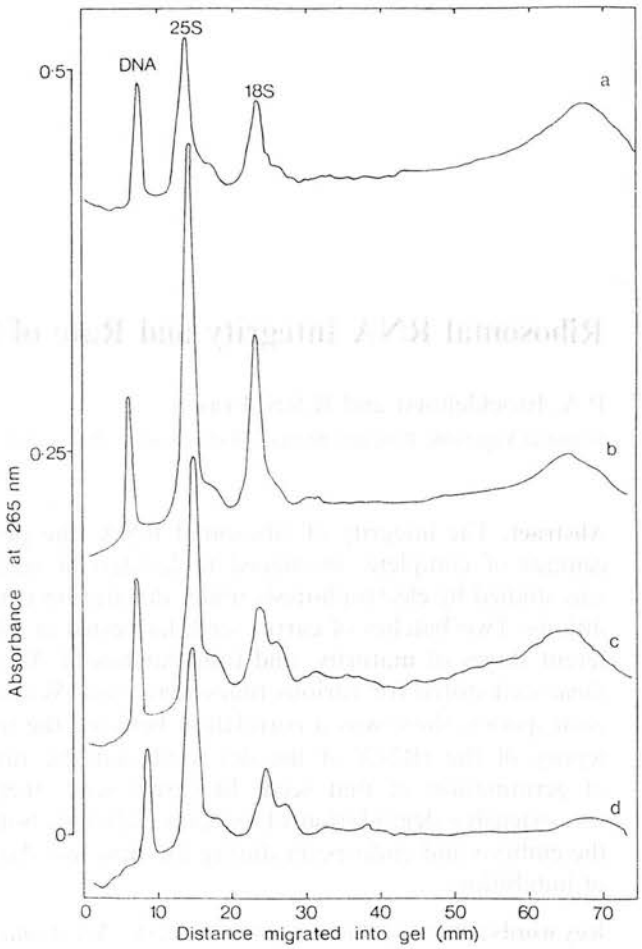

Fig. 1a–d. Aqueous (non-denaturing) polyacrylamide gel electrophoresis of nucleic acids from carrot seeds. **a** Dry, immature seed. **b** Dry, mature seed. **c** Immature seed, imbibed for 12 days. **d** Mature seed, imbibed for 12 days

In aqueous gels, rRNA molecules with internal breaks may migrate to the same position on the gel as intact molecules, because internal base-paired regions in the RNA keep the degradation products together during electrophoresis. Electrophoresis on formamide gels destroys this base pairing and exposes hidden nicks in the RNA. The advantage of the formamide technique over other denaturing techniques such as heating is that peaks of individual molecular species on gels are much sharper: minor differences in electrophoretic mobility caused by variation in secondary structure are abolished. This makes it possible to fractionate and recognise individual degradation products in complex mixtures.

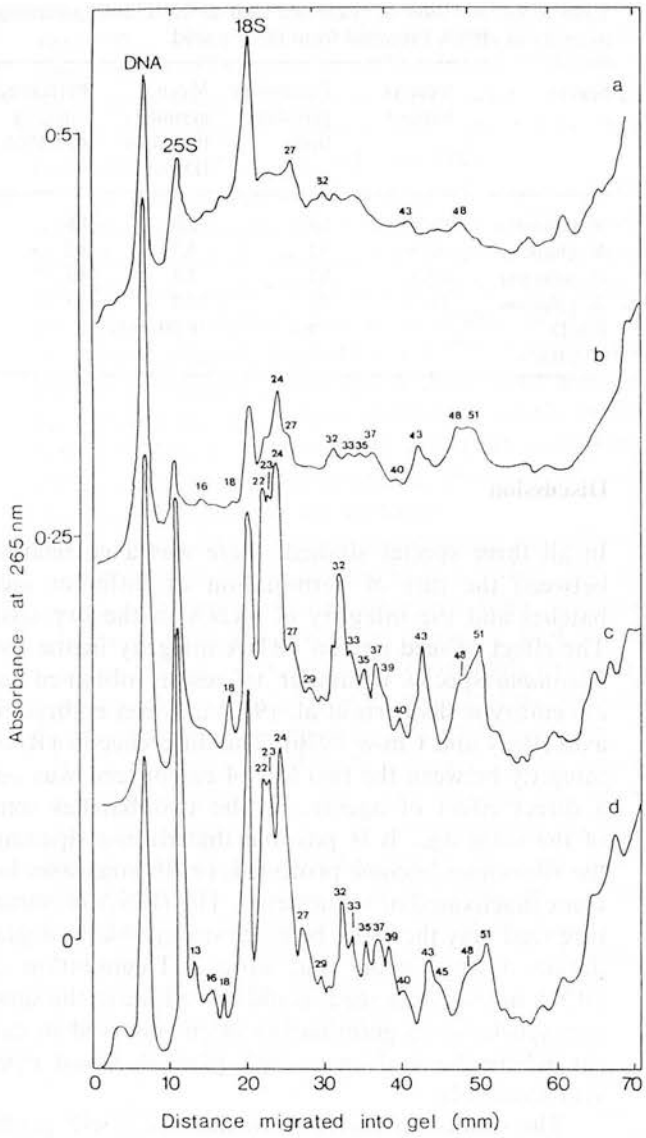

Fig. 2a–d. Electrophoresis under denaturing conditions on formamide-polyacrylamide gels of nucleic acids from carrot seeds. **a** Dry, immature seed. **b** Immature seed after 2 days imbibition. **c** Immature seed after 12 days imbibition. **d** Mature seed after 12 days imbibition

Electrophoresis of carrot seed nucleic acids on formamide gels revealed extensive degradation of the 25 *S* and 18 *S* rRNAs, and a large number of discrete, high molecular weight breakdown products, as well as highly mobile low molecular weight material. The pattern of peaks was quantitatively and qualitatively reproducible in replicate extractions and fractionations from each seed batch. The RNA from dry seed (Fig. 2a) showed some intact 25 *S* and 18 *S* forms; the ratio of 25 *S* to 18 *S* was however low, suggesting that the 25 *S* was proportionately more degraded. Discrete peaks of breakdown products in the high to middle molecular weight range (25 to 50 mm on the gel) were rare, compared with germinating seed (Fig. 2b, c). This suggests that if rRNA in the dry seed became nicked during development or storage of the seed, it was then more extensively degraded. Alternatively, the nicking may have been less site-specific in the dry seed than that which occurred during germination.

During the first two days of imbibition, there was further extensive degradation of 25 *S* and 18 *S* rRNAs, and peaks of degradation products in the middle to high molecular weight range appeared. These peaks are numbered indicating the distance migrated in mm to permit comparison between different gels. Later in germination (Fig. 2c), after considerable net accumulation of RNA had occurred (Table 1), more intact rRNA was present, together with considerable amounts of middle to high molecular weight degradation products.

Mature and immature seed showed similar patterns of breakdown products, in qualitative terms, both in the dry seed and during germination. For example, Fig. 2d shows that the pattern of degradation products in germinated, mature seed was very similar to that of comparable immature seed (Fig. 2c), indicating that the same sites in rRNA were susceptible to cleavage.

It is most likely that the nicking of rRNA occurred in vivo, and not during extraction of nucleic acids. Firstly, nucleic acids extracted from young tobacco leaves at the same time and under the same conditions showed very little evidence of degradation of 25 *S* and 18 *S* rRNAs. Secondly, the extraction procedure and all purification steps were in the presence of anionic detergents which are effective ribonuclease inhibitors.

To express the extent of degradation of rRNA at different stages of germination and in different seed batches in quantitative terms, we calculated the ratio of intact 25+18 *S* rRNA (measured on formamide gels) to total (intact plus degraded) 25+18 *S* rRNA. Total rRNA content was calculated by subtracting DNA and tRNA from total nucleic acid measurements, rather than by measuring 25 and 18 *S* rRNAs on aqueous gels, as under these conditions very extensive degradation of the rRNA can lead to loss of material from 25 and 18 *S* peaks.

Figure 3 shows changes in percentage integrity of the rRNA with time after the start of imbibition in the two batches of carrot seed. At no time was the percentage more than 44, and for most of the germination period only 25% or less of the rRNA was completely intact. In both batches of seed, there was a rapid decrease in integrity of the rRNA after imbibition. From day 2 to day 8, the percentage integrity increased in both batches (Fig. 3): this can only have

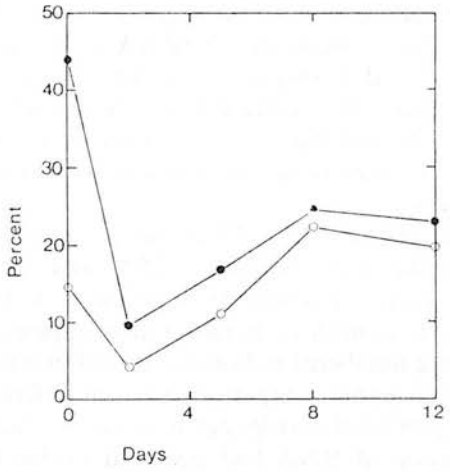

Fig. 3. Changes in the percentage integrity of rRNA with time from the start of imbibition of carrot seeds harvested while immature (○—○) or at maturity (●—●)

Table 2. Percentage integrity of rRNA extracted from immature carrot seed embryo and 'endosperm' fractions

	Period of imbibition at 10° C	
	2 h	48 h
Whole seed	13	6
Embryo	26	10
Endosperm + seedcoat	10	5

been the result of new synthesis of rRNA. The percentage of intact rRNA in the embryo and 'endosperm' fractions was roughly halved during the first two days of imbibition, just as in the whole seed (Table 2).

Dry, mature seed had a consistently higher proportion of intact rRNA than dry, immature seed, and this difference between the two batches persisted for at least 5 days after the start of imbibition (Fig. 3). These differences between the seed batches were reproducible in replicate experiments. There was thus a correlation between percentage integrity of the rRNA in dry seed and during the first 5 days of germination, and rate of germination (Table 1).

Nicotiana Seed

As further tests of the correlation between rate of germination and rRNA integrity, we examined seed of two *Nicotiana* species which had been stored for different times. Table 3 shows that for all seeds, the percentage final viability was high. However, in both species, germination of the seeds stored for several years was slower than for fresh seed. Seed with earlier germination had a higher percentage of intact rRNA in the dry seed than seed with slower germination.

Table 3. Germination of *Nicotiana* seed at 22° C and percentage integrity of rRNA extracted from the dry seed

Species	Year of harvest	Percentage germination	Mean germination time (Days)	Percentage integrity of rRNA
N. glutinosa	1978	98	2.8	88
N. glutinosa	1973	97	3.7	82
N. tabacum	1978	82	5.1	63
N. tabacum	1973	72	17.7	23
L.S.D. ($P \leq 0.05$)		5.3	0.59	–

Discussion

In all three species studied, there was a correlation between the rate of germination of different seed batches and the integrity of rRNA in the dry seed. The effect of seed age on rRNA integrity in the two *Nicotiana* species is similar to results obtained for rye embryos (Roberts et al. 1973) and pea embryonic axes (Bray and Chow 1976). The difference in rRNA integrity between the two lots of carrot seed was not a direct effect of ageing, as the two batches were of the same age. It is possible that during ripening, the ribosomes become protected, or ribonucleases become inactivated or sequestered. The rRNA of immature seed may therefore be more susceptible to degradation during drying and storage. Degradation of rRNA in immature seeds could contribute to the slow, non-synchronous germination often observed in carrot, where the seed on a single plant does not ripen synchronously.

The results for carrot show that seeds will germinate successfully with very extensively nicked rRNA. For much of the germination period, however, most of the rRNA was present as relatively large, discrete degradation products, which presumably were large enough to allow continued ribosome function.

Soon after imbibition of carrot seeds, there was a very extensive degradation of the dry seed rRNA to comparatively low molecular weight degradation products. The decline in integrity of embryo rRNA shows clearly that the early degradation is not confined to the storage tissue of the endosperm. We do not know exactly the extent to which rRNA can be degraded before ribosome function is lost, but these results suggest that the ribosomes of the dry seed might only be capable of protein synthesis for a short time after imbibition.

Although the changes in percentage integrity of rRNA in the embryo of carrot seed reflected early changes in the whole seed, rRNA from the embryo

was consistently less degraded than that of the 'endosperm'. This effect could possibly explain the higher levels of integrity of rRNA found in the *Nicotiana* seeds compared to the carrot seeds, since the embryo/'endosperm' ratio is much greater in *Nicotiana* than in carrot seed (Martin 1946).

The virtual absence of intact and large degradation products of carrot rRNA at 2 days, and the several-fold increase in total nucleic acid content during germination, suggest that the large rRNA degradation products so common later in germination (Fig. 2c) must have arisen by degradation of rRNA synthesized during germination. This point could not, however, be checked by radioactive labelling of newly synthesized rRNA, as carrot seeds failed to incorporate [^{3}H]uridine and [^{32}P]phosphate, probably because they failed to take up the isotopes.

The most likely mechanism causing the observed rRNA degradation is ribonuclease activity. The production of discrete and consistent high molecular weight degradation products suggests that certain sites on rRNA are particularly susceptible to endonucleolytic cleavage. Cleavage at specific sites could also occur during transcription and processing of the rRNA precursor, as double stranded rRNA regions and early assembly of ribosomal proteins with rRNA precursor would give rise to comparatively protected regions. Another possible cause of decreased rRNA integrity is the occurrence of DNA lesions in aged embryos (Cheah and Osborne 1978). However, it is difficult to see how DNA lesions, if spread at random through the multiple rRNA genes, could lead to sub-rRNA products of discrete size classes.

As a seed lot ages, its rate of germination declines before any loss of viability occurs (Delouche and Baskin 1973). Our results indicate that a reduction in rRNA integrity occurs at least as early as the decline in germination rate. The finding that loss of rRNA integrity precedes the decrease in protein synthetic activity of the ribosome (Bray and Chow 1976) also suggests that reduction in rRNA integrity may be initiated early in the ageing process. Thus measuring rRNA integrity of dry seeds may be a useful tool in the study of seed ageing and may provide a guide to possible future performance of different seed lots.

We thank Su Loughlin and Jane Mills for technical assistance, and David Gray for carrot seed.

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Received 1 August; accepted 24 November 1979

Reprinted from Trends in Biochemical Sciences – October, 1980 (pp. II/III)

Why bank plant genes?

Plant breeding provides modern agriculture with crop varieties having commercially desirable features such as yield, disease resistance and uniformity. Breeding has two opposite effects on the total genetic range of a crop. Variability is increased by hybridization and introduction of genes from related wild species. In contrast, older varieties without the currently required combination of characteristics are not grown and quickly become extinct. This reduces genetic range: genes which could be of importance for future breeding are lost. For example, varieties of cauliflower with resistance to ringspot disease were allowed to become extinct 30 years ago, and today no gene for this resistance is available.

To preserve the maximum range of genes for future vegetable breeding, a gene bank is being established at the National Vegetable Research Station, Wellesbourne, U.K. I shall describe how the bank will operate, and suggest that it provides opportunities for research in biochemistry and related disciplines of mutual benefit to biochemists and breeders.

The Wellesbourne gene bank will be built and supported for seven years with a special £700,000 fund raised internationally by the famine relief organization Oxfam. Thereafter, it will be financed by the U.K. Agricultural Research Council. Oxfam's support reflects the importance of vegetable crops in developing countries: conservation and exploitation of vegetable genetic resources in breeding programmes could contribute significantly to solving the nutritional problems of the third world. Physically, gene banking involves storage of up to 1 l of specially dried seed of each variety at −20°C. Vegetable seeds can generally be stored under such conditions for 30–50 years without serious loss of viability. Seed of each lot will periodically be removed from store for regeneration of fresh seed. The bank will store about 3,000 temperate and 3000 tropical vegetable varieties, as well as wild species which may be useful for breeding purposes. The Wellesbourne bank is the first to concentrate entirely on vegetables. In other countries, there are banks for cereals and staple crops such as beans. Some have limited collections of particular vegetable crops, or of varieties from limited geographical areas. The Wellesbourne bank will exchange varieties and information with other banks; seed of important varieties will be stored in duplicate in different parts of the world to guard against accidental loss. For instance, Wellesbourne will hold in duplicate the 3000 varieties of tropical vegetables in the Asian Vegetable Research Centre Gene Bank in Taiwan and the collection of the Indian Bureau of Genetic Resources.

For its primary role as a source of genes for breeding, bank size is important. Therefore as many varieties as possible will quickly be taken into store. Storage will be non-discriminatory: any present day variety may contain genes which could be of use in the future.

A source, to be useful, must be able to be tapped efficiently. All available information about each variety will be stored on computer file to aid selection and retrieval. Breeders will be able to request seed with genes for particular characteristics or combinations of characteristics. To encourage the full use of the bank's facilities by breeders and scientists, its services will be free.

While the implications of the gene bank for biochemically related research are less immediately obvious, there are several areas where the gene resources and information stored could help the biochemist. Conversely, knowledge derived from biochemical experiments on gene bank material will undoubtedly help the bank in the long term with its primary aim of assisting breeding.

As a source of material of different varieties, often with known history and relationships, the bank will be invaluable for studies in comparative biochemistry, molecular evolution and biochemical taxonomy. Attention in breeding is increasingly being given to biochemical characteristics, such as amino acid composition of proteins, and lectin content. Moreover, biochemical methods such as isoenzyme tests are finding use in breeding and in differentiation of new varieties. Thus information from biochemical studies, if recycled to the gene bank data-base, will be of use to breeders interested in these aspects.

For investigations of gene action, metabolic pathways, differentiation and morphogenesis, it is useful to have wild type and mutant alleles of relevant genes in an otherwise uniform genetic background. Near isogenic lines suited to such studies have been produced: a notable example is the tomato series of over 100 mutant alleles transferred to a common genetic background by L. A. Darby and coworkers at the Glasshouse Crops Research Institute. Deposition of such lines with a central gene bank would increase their availability to biochemists.

Many plant varieties are resistant to specific pests and diseases, but generally the biochemistry of the action of resistance genes is not understood. Studies of resistance biochemistry will benefit from the gene bank as a source of susceptible and resistant varieties. The understanding gained should help in future breeding for resistance, and in designing new chemical methods of disease control.

The biochemistry of seed dormancy, germination, ageing and loss of viability require further study. The bank can assist by providing seed of known ages and storage history. More biochemical understanding of these processes may lead to the development of better gene-banking techniques.

Gene banks are planned to have a long lifespan. Early in that lifespan, somatic hybridization and genetic engineering will probably begin to allow transfers of genetic material at present made impossible by barriers to normal hybridization. These biochemical techniques could instigate a new era in plant breeding. But genetic engineering cannot yet create useful genes *de novo*. The gene bank, by preserving a wide selection of existing genes and gene combinations, will provide an essential foundation for a genetic engineering approach to breeding new varieties.

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Molec. Gen. Genetics 106, 73—79 (1969)

Effects of Two TMV Strains on the Synthesis and Stability of Chloroplast Ribosomal RNA in Tobacco Leaves*

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Received November 5, 1969

Summary. Multiplication of TMV-strains *vulgare* (light-green/dark-green mosaic symptoms) and *flavum* (severe yellow/green mosaic) had different effects on the ribosomal RNA of tobacco leaf chloroplasts. *Vulgare* inhibited chloroplast ribosomal RNA synthesis while having no effect on cytoplasmic ribosomal RNA synthesis (Fig. 2). *Flavum* inhibited chloroplast ribosomal RNA synthesis more severely than *vulgare*, and caused an earlier degradation of chloroplast ribosomal RNA than in control or *vulgare*-infected leaves (Fig. 1). *Flavum* also inhibited cytoplasmic ribosomal RNA synthesis. A connection between these differing effects on chloroplast ribosomal RNA metabolism and severity of visible symptoms is suggested, and discussed in relation to a possible influence on symptoms of denatured virus coat protein.

Introduction

Hirai and Wildman (1969) reported recently that TMV strain U1 (*vulgare*) inhibited chloroplast ribosomal RNA synthesis while not affecting cytoplasmic ribosomal RNA synthesis. Protein synthesis by chloroplasts isolated from virus-infected leaves was found to be inhibited. They suggested that these effects on chloroplast metabolism give rise to the light-green mosaic symptoms of infection.

In view of the very different visible symptoms produced by different strains of TMV, it is of interest to compare the consequences of their multiplication for chloroplast metabolism. I have examined the effects of infection by TMV strains *vulgare* (light-green symptoms) and *flavum* (severe yellowing and early leaf collapse) on chloroplast ribosomal RNA synthesis and stability. The results suggest a correlation between the effects of virus multiplication on chloroplast ribosomal RNA synthesis and degradation, and the severity of visible symptoms.

Materials and Methods

Tobacco (*Nicotiana tabacum*) var. "Samsun" plants were grown in a greenhouse at $23 \pm 2°C$ with continuous illumination of 3,500 lux night (Osram L-Fluora tubes) and *ca.* 7,500 lux day. 9 cm long leaves on 20 cm-tall plants were dusted with carborundum and inoculated by rubbing with either sterile phosphate buffer, 0.066 M, pH 7.0 (Control); 0.1 % TMV-strain *vulgare* in phosphate buffer, or TMV-strain *flavum*-infected leaves ground in phosphate buffer. The leaves were washed with running tap-water immediately after inoculation.

Radioactive Incubation. A detached leaf was placed with its petiole in 0.5 ml water containing 0.5 mc ^{32}P-phosphate. This was completely taken up in 60—90 minutes. The leaf was then floated on water until a total ^{32}P incubation time of 5 hours was reached. This method ensured equal uptake of ^{32}P by all treatments.

* Abbreviations: TMV = Tobacco Mosaic Virus; RNA = Ribonucleic acid; DNA = Deoxyribonucleic acid; m = millions (in molecular weight values).

Bacterial ribosomal RNAs and chloroplast ribosomal RNAs have similar electrophoretic mobilities (Loening and Ingle, 1967). Although the ^{32}P incubations were done under non-sterile conditions, I do not think that labelling of bacterial ribosomal RNA influenced the labelling pattern of chloroplast ribosomal RNA: when old tobacco leaves (which do not synthesise chloroplast ribosomal RNA) were labelled in this way, no radioactivity was found in the bacterial/chloroplast ribosomal RNA position.

RNA Extraction. 0.4 g leaf material was homogenised for 10 seconds in a Bühler Homogeniser (E. Bühler, Tübingen, Germany) at *ca.* 40,000 r.p.m. in 8 ml 30 mM tris-chloride, pH 7.6, containing 0.5 % 1,5-naphthalenedisulphonic acid, disodium salt (Eastman Kodak) (Hastings and Kirby, 1966) and 5 mM Cleland's Reagent, at 0°C. Immediately after homogenisation, sodium tri-iso-propylnaphthalenesulphonate (Eastman Kodak) and sodium 4-aminosalicylate were added to final concentrations of 1 and 5 % respectively (Parish and Kirby, 1966). The homogenate was shaken with an equal volume of phenol-cresol mixture (Kirby, 1965: redistilled phenol 1,000 g; redistilled m-cresol 140 ml; 8-hydroxyquinoline 1 g; water to saturate). Thereafter extraction was at 10°C. The aqueous phase was recovered by centrifuging for 20 minutes at 10,000 × g. Sodium chloride was added to a final concentration of 0.3 M and the phenol-cresol extraction repeated twice. Nucleic acids were precipitated from the final aqueous phase by addition of two volumes of ethanol and 12 hours at −20°C. The nucleic acids were collected by low-speed centrifugation and dissolved in 2 ml 0.15 M sodium acetate pH 6 containing 0.5 % sodium dodecyl sulphate, and were dialysed against this solution for 24 hours at room temperature. Nucleic acids were precipitated by ethanol at −20°C and the precipitate was washed with ethanol, dried and dissolved in 0.2—1.0 ml electrophoresis buffer containing 5 % sucrose.

Electrophoresis. Fractionation of nucleic acids was on polyacrylamide gels (Loening, 1967), 7 cm long, 6 mm diameter, containing 2.4 % acrylamide and 0.12 % bisacrylamide. The buffer had a pH of 7.8 and contained tris 36 mM; NaH_2PO_4 30 mM; EDTA 1 mM and 0.2% sodium dodecyl sulphate. About 15 µg of RNA in 10 µl electrophoresis buffer were applied to each gel. Electrophoresis was for 2.5 hours at room temperature at 50 V and 5 mA per gel. The gels were scanned optically at 265 nm in a Joyce-Loebl Chromoscan. Radioactive gels were then frozen in solid CO_2 and sliced transversely at 0.5 mm intervals with a Mickel Gel Slicer (Mickel Laboratory Engineering Co., Gomshall, Surrey, England). The slices were dried on a film base and counted in a Packard 460 chromatogram scanner.

Chlorophyll. Chlorophyll content was determined by the method of Comar and Zscheile (1942). 1 g of leaf was homogenised in the Bühler homogeniser in 8 ml 80 % acetone. The pigment was extracted into diethyl ether and chlorophyll concentration calculated from the absorbances at 600, 644 and 662 nm.

Results

Changes in Chloroplast Ribosomal RNA in Control Leaves

Electrophoresis of nucleic acids from young (5 cm long) control leaves (Fig. 1 a) shows DNA at 0.8 cm; 1.28 m[1] and 0.71 m cytoplasmic ribosomal RNA at 2.5 and 3.8 cm and 1.1 m and 0.56 m chloroplast ribosomal RNA at 2.9 and 4.4 cm. The chloroplast RNA species were present in approximately equimolar amounts: this contrasts with previous reports on other tissues, such as French bean and raddish (Loening and Ingle, 1967; Ingle, 1968) and spinach, pea, tomato and tobacco (Spencer and Whitfeld, 1966) where the 1.1 m component was always reduced or absent.

[1] Bishop, Claybrook and Spiegelman (1967) and Loening (1969) have shown a linear relationship between log molecular weight and electrophoretic mobility. I will refer to all RNA species by molecular weight values determined by reference to the plant cytoplasmic ribosomal RNAs, and using Loening's (1969) molecular weight values for these molecules of 1.28 m and 0.71 m.

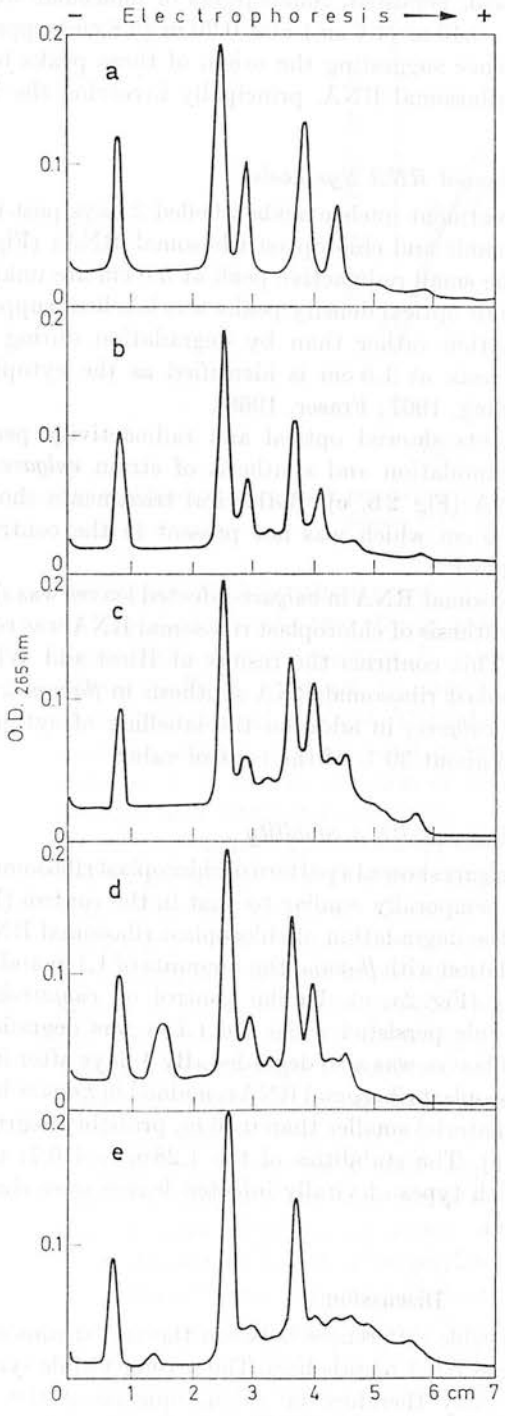

Fig. 1

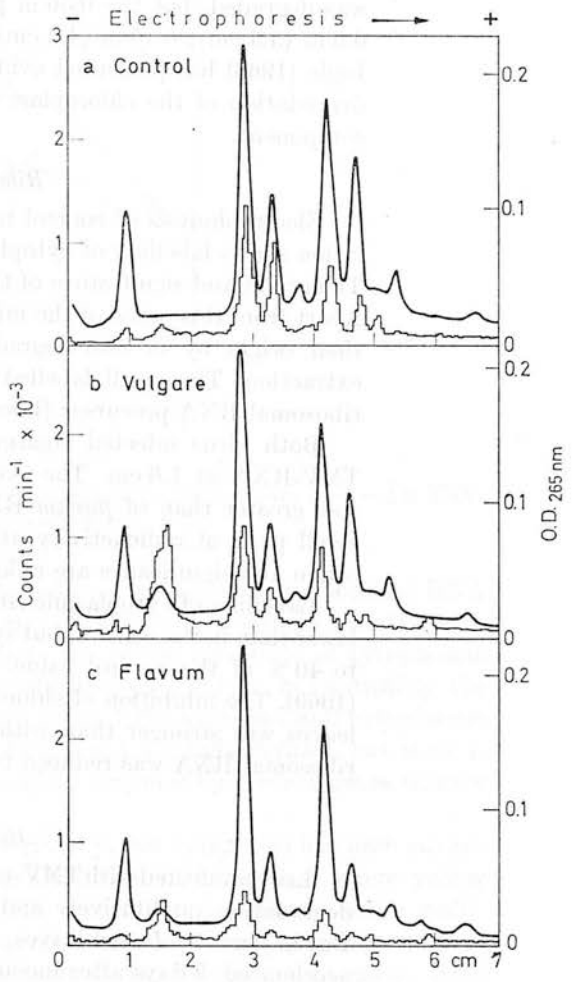

Fig. 2

Fig. 1 a—e. Electrophoresis of nucleic acids from tobacco leaves. a 5 cm long healthy leaf. b 9 cm long healthy leaf, at time of inoculation. c Control leaf, 17 cm long, 4 days after sham inoculation of 9 cm leaf. d Leaf 4 days after inoculation with TMV-strain *vulgare*. e Leaf 4 days after inoculation with TMV-strain *flavum*

Fig. 2 a—c. Electrophoresis of tobacco leaf nucleic acids labelled with ^{32}P and extracted 2 days after inoculation with a Control; b TMV-*vulgare*; c TMV-*flavum*

During growth of the leaf (Fig. 1 b, c) the 1.1 m chloroplast ribosomal RNA was degraded, but the 0.56 m peak persisted. Small peaks of molecular weights 0.9 m (3.3 cm); 0.45 m (4.2 cm); 0.40 m (4.6 cm) and 0.20 m (5.8 cm) appeared. Ingle (1968) has presented evidence suggesting the origin of these peaks by the degradation of the chloroplast ribosomal RNA, principally involving the larger component.

Ribosomal RNA Synthesis

Electrophoresis of control treatment nucleic acids labelled 2 days post-inoculation shows labelling of cytoplasmic and chloroplast ribosomal RNAs (Fig. 2a) The origin and significance of the small radioactive peak at 5.0 cm are unknown Apart from this, none of the minor optical density peaks was labelled, supporting their origin by *in vivo* degradation rather than by degradation during RNA extraction. The small labelled peak at 1.5 cm is identified as the cytoplasmic ribosomal RNA precursor (Loening, 1967; Fraser, 1968).

Both virus infected treatments showed optical and radioactivity peaks of TMV-RNA at 1.6 cm The accumulation and synthesis of strain *vulgare* RNA was greater than of *flavum* RNA (Fig 2b, c). Both viral treatments showed a small peak of radioactivity at 6 cm which was not present in the control. Its origin and significance are unknown.

Labelling of cytoplasmic ribosomal RNA in *vulgare*-infected leaves was slightly lower than in the control, but synthesis of chloroplast ribosomal RNA was reduced to 40 % of the control value. This confirms the results of Hirai and Wildman (1969). The inhibition of chloroplast ribosomal RNA synthesis in *flavum*-infected leaves was stronger than with *vulgare*; in addition the labelling of cytoplasmic ribosomal RNA was reduced to about 30 % of the control value.

Ribosomal RNA Stability

Leaves infected with TMV-*vulgare* showed a pattern of chloroplast ribosomal RNA degradation qualitatively and temporally similar to that in the control (Fig. 1). But in *flavum*-infected leaves, the degradation of chloroplast ribosomal RNA was accelerated. 2 days after inoculation with *flavum*, the amounts of 1.1 m and 0.56 m were lower than in the control (Fig. 2a, c). Unlike control or *vulgare*-infected leaves, where the 0.56 m molecule persisted while the 1.1 m was degraded, the 0.56 m RNA in *flavum*-infected leaves was also degraded. By 4 days after inoculation, little 1.1 m and 0.56 m chloroplast ribosomal RNA remained in *flavum*-infected leaves, and a large amount of material smaller than 0.56 m, probably degradation products, was present (Fig. 1 e). The stabilities of the 1.28 m and 0.71 m cytoplasmic ribosomal RNAs in both types of virally infected leaves were similar to those in the control (Fig. 1 c—e).

Discussion

There are therefore considerable differences between the two strains of TMV in effect on chloroplast ribosomal RNA metabolism. The severer visible symptoms produced in *flavum* infection may therefore be a consequence of the severer inhibition of chloroplast ribosomal RNA synthesis (and possibly other types of

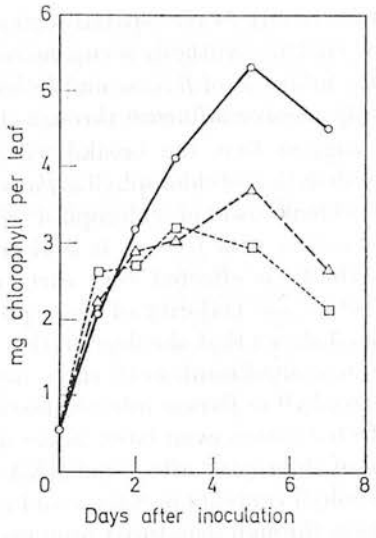

Fig. 3. Chlorophyll content per leaf after inoculation with TMV-*vulgare* (△——△); TMV-*flavum* (□- - - -□); control (○——○)

chloroplast RNA synthesis), and the degradation of chloroplast ribosomal RNA by this strain, as the two effects must lead to a virtual cessation of protein synthesis inside the chloroplasts. In addition, the severe inhibition of cytoplasmic ribosomal RNA synthesis by *flavum* might also play a part in controlling the stability of the chloroplasts. The role of nuclear RNA synthesis and cytoplasmic protein synthesis in chloroplast metabolism is not yet fully defined, but there is some evidence for specification of chloroplast enzymes by nuclear genes (review by Kirk and Tilney-Basset, 1967).

An alternative explanation for the origin of yellow symptoms has been advanced by Jockusch and Jockusch (1968). They demonstrated that where yellow symptoms were produced, either by yellow mutants such as *flavum* or by multiplication of TMV mutants with temperature-sensitive coat proteins at high temperatures, greater amounts of insoluble virus coat protein were present. They argued that the insoluble proteins may lead to disruption of cell organelles, in the case of chloroplasts liberating chlorophyllase from some bound state and leading to chlorophyll breakdown and yellowing. This hypothesis might be extended to explain the accelerated breakdown of chloroplast ribosomal RNA in *flavum*-infected leaves by liberation of ribonuclease in disrupted chloroplasts.

However, there are certain arguments against the liberation of spatially separated enzymes through disruption of structure by denatured proteins being the sole cause of yellow symptoms.

1. Measurements of chlorophyllase activity (Peterson and McKinney, 1938; G. Paulsen-Oehlen, unpublished data) show that *flavum*-infected leaves attain a much higher chlorophyllase activity than control or *vulgare*-infected leaves. Clearly it is important to distinguish between whether this increased activity is a result of liberation and activation of pre-existing enzyme, or stimulation of enzyme synthesis. As the assays were done on acetone-dry powder extracts, which

would tend to eliminate any *in vivo* spatial segregation of enzyme and substrate, stimulation of new enzyme synthesis seems more probable. This would argue in favour of a directive influence of *flavum* multiplication on chloroplast metabolism rather than a merely passive influence through disruption of structure.

2. My results suggest that the breakdown of chloroplast ribosomal RNA begins much earlier than that of chlorophyll in *flavum*-infected leaves. From Fig. 2c, where about 50% breakdown of chloroplast ribosomal RNA is recorded by 48 hours after inoculation with *flavum*, it is clear that the effect on chloroplast ribosomal RNA stability is effected very early in virus multiplication, indeed must be effected before the majority of virus protein has been synthesised. On the other hand, Fig. 3 shows that the degradation of chlorophyll in virus-infected leaves did not become significant until three days after inoculation, and that degradation of chlorophyll in *flavum*-infected leaves first became more pronounced than in *vulgare*-infected leaves even later. Some degree of simultaneity of inception of degradation of chloroplast ribosomal RNA and chlorophyll, and an earlier distinction in chlorophyll contents in *flavum* and *vulgare*-infected leaves might be expected if disruption through denatured proteins were the sole cause.

3. The TMV mutant Ni 118 has temperature-sensitive coat protein (Jockusch, 1968), but produces green symptoms at high temperatures.

In view of these doubts, experiments are being carried out in an attempt to discover the relative importance of the effects on RNA synthesis and stability and the effects of denatured proteins in the determination of visible symptoms.

Acknowledgements. I thank Prof. G. Melchers, Dr. S. Sarkar and other colleagues in Tübingen for advice and discussions, and Miss U. Kloepfer for capable technical assistance.

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Communicated by H. G. Wittmann

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Universitätsdruckerei H. Stürtz AG Würzburg
Printed in Germany

Reprinted from VIROLOGY, Volume 45, No. 3, September 1971

VIROLOGY **45**, 804–807 (1971)

Extraction and Assay of TMV RNA

Available physical/chemical methods for estimation of TMV concentrations in infected plants (*1*, *2*) suffer from low sensitivity, interference by contaminants, and dependence on virus particle stability. I report here an accurate and sensitive TMV assay involving extraction and measurement of TMV RNA.

TMV RNA is clearly separated from host plant nucleic acids by polyacrylamide-gel electrophoresis (Fig. 1A). The peak area of a nucleic acid type on the ultraviolet absorption scan of the gel is linearly proportional to the amount of nucleic acid loaded onto the gel. By electrophoresis of known amounts of highly purified tRNA (Schwarz Bioresearch) the peak area/weight of nucleic acid ratio was determined. This permits the calculation of the weight of TMV RNA, or any other RNA type, on the gel.

To use this as an assay method, undegraded nucleic acids must be extracted in high, reproducible yields from the plant. A method based on those of Kirby *et al.* (*3*, *4*) was found suitable. One-half gram of TMV-infected tobacco leaf was homogenized at 0° in at least 15 vol of homogenizing medium (Tris–HCl 30 m*M*, pH 7.8; sucrose 0.3 *M*; KCl 50 m*M*; $Mg(CH_3COO)_2$ 1 m*M*; dithiothreitol 5 m*M*) for 30 seconds at 40,000 rpm in a rotating-knife type homogenizer. Extraction of undegraded nucleic acids from extremely old leaves was facilitated by 1% diethyl pyrocarbonate (*5*) in the homogenizing medium. Immediately after homogenization, sodium triisopropylnaphthalene sulfonate and sodium 4-amino-salicylate were added to final concentrations of 1 and 5%, respectively. The homogenate was deproteinized by shaking with a phenol/*m*-cresol mixture (*4*) at 20° and was centrifuged for 10 minutes at 10,000*g*. Washing of the phenol layer with a further 3 ml of homogenizing medium plus detergents released 15% more nucleic acid. The combined aqueous layers were made to 0.3 *M* with NaCl and were extracted twice more with phenol/cresol. Nucleic acids were precipitated by 70% ethanol at −20°, and then dissolved in 1% sodium dodecylsulfate, 0.15 *M* sodium acetate, pH 6.0. Dialysis against this solution for 24 hours at room temperature effectively removed contaminants interfering with RNA fractionation on gels. The nucleic acids were reprecipitated by ethanol at −20°, dissolved in Tris–phosphate electrophoresis buffer (*6*) and fractionated on polyacrylamide gels as described by Loening. (*6*). The total nucleic acid extracted by the detergent–phenol method was calculated from the ultraviolet absorption spectrum of a sample of the solution prepared for electrophoresis hydrolyzed with 0.5 *N* $HClO_4$ for 20 minutes at 70°.

Points of importance for TMV RNA extraction are: (1) A more mobile shoulder, indicating degradation, appeared on the TMV RNA peak when detergents were present during the homogenization (Fig. 1B). This also occurred with gentler methods of homogenization. TMV RNA seems to be especially sensitive to damage by shearing forces when dissociated from its protein. (2) Outside the pH range 7.5–8.0, differential failure to extract specific nucleic acids could occur. (3) The total yield of nucleic acid increased with the temperature used for phenol extractions (range 0–60°), but above 20° degradation occurred, to which TMV RNA was especially sensitive.

To evaluate the yield of the detergent–phenol extraction, the total nucleic acid content of the leaf had to be measured. After acid and organic-solvent washes (*7*) nucleic acids were extracted by hot $HClO_4$ (*8*) and their concentration calculated from the ultraviolet absorption spectrum. The spectra obtained, and also those of detergent–phenol-extracted nucleic acids, showed 260:235 nm absorbance ratios of about 2.5:1, and were thus clean enough for nucleic acid measurement by absorbance at 260 nm.

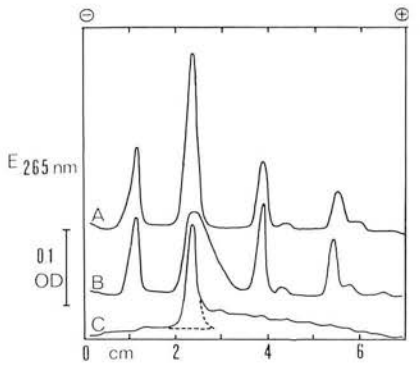

FIG. 1. Ultraviolet absorption scans (Joyce Loebl, Chromoscan) of electrophoresis on polyacrylamide gels of nucleic acids extracted from (A) 20-cm long leaves of *Nicotiana tabacum* var. Samsun, 15 days after infection by TMV strain *vulgare*. Nucleic acid extraction as described in the text. B. As A, except that 1% triisopropylnaphthalene sulfonate and 5% 4-amino-salicylate were present during homogenization. C. RNA from purified TMV. The broken line shows the delimitation of the monodisperse TMV RNA peak used to determine the percentage of TMV RNA present as the intact molecule. 2.2% acrylamide gels. Electrophoresis for 3 hours at 50 v. The peaks are: at 1.0 cm, DNA; at 2.5 cm, TMV RNA; at 3.9 and 5.4 cm, ribosomal RNA; and at 4.4 and 5.9 cm, chloroplast ribosomal RNA.

With the same infected tobacco leaves as used for Fig. 1A, the total nucleic acid content was found to be 1106 ± 19 μg/g leaf (mean ± SE; eight determinations). The detergent–phenol method extracted 886 ± 19 μg/g, a yield of 80%. Measurement by peak areas of the weights of all individual nucleic acid types on the gels (DNA, TMV RNA, cytoplasmic and chloroplast ribosomal RNA,[1] tRNA, 5S RNA) accounts for 784 ± 18 μg/g, i.e., 89% of the yield by detergent–phenol extraction. This suggests that the nucleic acids were extracted essentially without degradation. The sharp, monodisperse TMV RNA peak confirms this by the absence of a light shoulder of more mobile degradation products (Fig. 1A). Further tests of yield using solutions of purified TMV showed that the detergent–phenol method extracted 94% of total RNA from TMV. Ninety per cent of virus RNA (measured by gel peak areas) was recovered when nucleic acids were extracted from 6 mg virus in the presence of 0.5 g healthy tobacco leaf. The detergent–phenol extraction therefore provides the high, reproducible yields of undegraded RNA required for the gel assay.

In an experiment comparing gel assay with TMV assay by isolation of virus particles by heat treatment and centrifugation and their estimation by UV absorption (*2*), TMV concentration was found to be 5.8 mg/g leaf by gel assay (20 × 290 ± 16 μg TMV RNA/g leaf). The heat/centrifugation result (5.4 ± 0.4 mg TMV/g leaf) was in good agreement. The maximum concentrations of TMV in leaves detected by gel assay were 10–12 mg/g, similar to the maximal amounts reported by other chemical assays (*1*, *2*).

Table 1 shows estimation of leaf TMV concentration by infectivity, by comparison of the number of lesions produced by the infected leaves with the numbers produced by purified virus solutions of known concentrations, and compares the results with gel assay determinations. Figure 1C shows that not all of the RNA of purified virus is complete TMV RNA; a tail of smaller degradation products is also present. In freshly prepared TMV samples, up to 80% of the RNA is intact; the percentage falls with storage, and with freezing and thawing (Table 1). The lower the integrity of the TMV RNA of the standard virus solution, the higher the calculated leaf virus concentration. Even with 80% of the standard TMV RNA intact, the infectivity assay of leaf virus concentration gave a value 7 times that obtained by parallel gel assay (Table 1), clearly much too high. Mundry (*2*) also found a large discrepancy between TMV concentrations determined chemically or by infectivity. Purification might reduce the specific infectivity of virus particles, but this cannot involve more than a minute change in the RNA, as over 80% found on gels had the full

[1] The nucleic acids of Fig. 1A were extracted from aged leaves, which have low chloroplast ribosomal RNA contents. The detergent–phenol method described will extract chloroplast ribosomal RNA successfully from younger tobacco leaves and gives a 2:1 ratio of 1.1×10^6 dalton:0.56×10^6 dalton components (*11*).

TABLE 1

DETERMINATIONS OF TMV CONCENTRATION OF INFECTED TOBACCO LEAVES BY INFECTIVITY AND BY GEL ASSAY

	Infected tobacco leaves[a]	Reference standards: Purified TMV solutions			
		1	2	3	4
Dilution	10^7	5×10^6	5×10^6	5×10^6	10^6
Lesions/leaf[b]	77 ± 7[c]	206 ± 27	200 ± 30	194 ± 34	60 ± 8
μg TMV/ml[d]		0.012	0.012	0.012	0.036
Leaf virus concentration calculated with respect to each reference TMV solution (mg TMV/g leaf)		43.3	44.7	46.0	462.0
Storage time of standards at −20°		Fresh	Fresh	1 Year	1 Year
Times thawed and refrozen		0	0	0	10
% of RNA in standard found to be in complete TMV RNA molecules[e]		79	83	59	17
TMV RNA (μg/g leaf) found by detergent–phenol extraction and gel assay	313 ± 6[c]				
Equals TMV (mg/g) (20 × RNA value)	6.26				

[a] Leaves from *N. tabacum* var. Samsun plants 14 days after infection with TMV strain *vulgare*.

[b] At least 10 leaves of *N. tabacum* var. Xanthi-nc were inoculated with each sample.

[c] Standard error of the mean.

[d] Calculated from the absorbance of the virus solution at 260 nm; 1 mg TMV/ml = 2.7 OD.

[e] Amount of complete TMV RNA derived from peak area of monodisperse TMV RNA peak on gel (cf. Fig. 1C). Total RNA content of the virus RNA preparation calculated from the UV spectrum of a $HClO_4$ digest.

molecular weight (Table 1). Alternatively, the TMV in the leaf might be activated in some way. Stimulation of infectivity of purified viruses by leaf homogenates has been reported (*9*, *10*). Infectivity seems therefore unreliable for estimation of absolute virus concentrations. Possible influences on the specific infectivity of the virus of plant age, virus strain and any experimental treatment mean that even *comparative* estimates of virus concentration by infectivity should be supported by chemical measurements.

The lower limit of sensitivity of the gel assay method is equivalent to 2 μg virus/g leaf. Figure 2A shows the detection of 0.1 μg TMV RNA in the presence of 167 μg host nucleic acid, equivalent to 8 μg virus/g leaf. Reproducible estimates of leaf virus concentration can be obtained at this level. Thus the gel assay method is much more sensitive than other physical/chemical methods. Heat precipitation–centrifugation methods (*1*, *2*) are unreliable below about 0.5 mg virus/g. On the other hand, infectivity can *detect* TMV concentrations about 10^{-3} of the gel assay minimum. But by radioactive labeling of the infected material, the sensitivity of the gel method for detection of TMV RNA synthesis is greatly increased. Figure 2C shows TMV RNA label detected in a 2-mg piece of cultured root-tip, in which the maximum TMV concentration reached was under 20 μg/g. It is possible to detect and measure rates of virus RNA synthesis in individual segments of root as short as 200 μ (200 μg weight). This permits, for example, a precision of analysis of location of TMV synthesis greater than that attainable by infectivity.

Figure 2B shows detection of viral nucleic acid, by the gel method, in leaves infected with a protein-defective mutant. The method has also been used to estimate TMV RNA synthesis of temperature-sensitive pro-

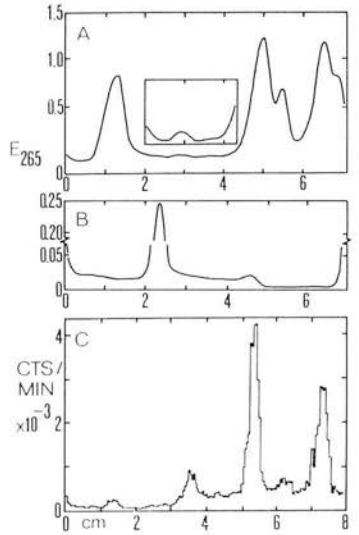

FIG. 2. Detection and measurement of low concentrations of TMV by the gel assay method, and detection of TMV RNA synthesis in minute amounts of tissue. A. 0.1 μg TMV RNA (at 3 cm) fractionated in the presence of 167 μg leaf nucleic acid. The inset shows the region from 2.0 to 4.2 cm scanned with 8 times increased vertical sensitivity. 2.2% gel; 4 hours electrophoresis. B. Electrophoresis of nucleic acids from tobacco leaves 5 days after infection with the protein-defective TMV strain DT_2. (Infected leaves provided by Dr. S. Sarkar.) 2.1% gel run for 4 hours. TMV RNA at 4.6 cm. C. Electrophoresis of ^{32}P-labeled nucleic acids extracted from a 2-mg segment of a TMV-infected cultured tomato root, after incubating for 1 hour with ^{32}P-phosphate and 7 hours with nonradioactive phosphate. Counting of gels as described in Ref. (*11*). The labeling peak at 3.6 cm is TMV RNA; those at 5.2 and 7.3 cm are ribosomal RNA. 2.2% gel run 4 hours.

tein mutants of TMV grown at high temperatures (I. Takebe, personal communication). Thus the gel assay procedure seems to be suitable for use with various types of TMV strains, independent of the physical nature and biological behavior of the particle.

ACKNOWLEDGMENTS

This work was supported by the Max-Planck-Gesellschaft zur Förderung der Wissenschaft. I thank Prof. G. Melchers for encouragement and Frl. Ute Klöpfer for capable technical assistance.

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Accepted May 24, 1971

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Reprinted from VIROLOGY, Volume 47, No. 2, February 1972

VIROLOGY **47**, 261–269 (1972)

Effects of Two Strains of Tobacco Mosaic Virus on Growth and RNA Content of Tobacco Leaves

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Accepted September 10, 1971

Tobacco leaves 1.5 to 18 cm long were infected with tobacco mosaic virus (TMV) strains *vulgare* or *flavum*. Nucleic acids were extracted, and fractionated and assayed by gel electrophoresis. Leaf growth and contents of DNA, ribosomal RNA, chloroplast ribosomal RNA, transfer RNA, and TMV-RNA were measured at various times after infection.

The younger the leaf infected, the longer the duration of synthesis of TMV-RNA, and the higher the final virus concentration reached. Synthesis of *flavum*-RNA was as great as that of *vulgare*-RNA in young leaves, but was much less than that of *vulgare*-RNA in old leaves.

The growing, healthy leaf accumulated ribosomal RNA, chloroplast ribosomal RNA, and transfer RNA. The maximum contents of these per leaf were reached as or just before the leaf reached its maximum length of 15–20 cm. Thereafter there was a loss of these components.

TMV-infection of a 1.5 cm-long leaf inhibited its growth and nucleic acid accumulation. Infection of a 5 cm-long leaf had no effect on the subsequent rises in ribosomal RNA and transfer RNA contents, but chloroplast ribosomal RNA accumulation was inhibited. The effects of TMV-strains *vulgare* and *flavum* on young leaves were similar.

Infection of older leaves, at or later than the time of maximum ribosomal RNA content, led to contents of ribosomal and transfer RNA higher than in control leaves, as the rate of loss of these two components was reduced, especially by strain *flavum*. Under no conditions was a degradation of cytoplasmic ribosomal RNA found as a result of viral infection, but chloroplast ribosomal RNA was quickly degraded in *flavum*-infected leaves.

INTRODUCTION

Tobacco mosaic virus (TMV) must be judged one of the most successful of plant viruses in terms of the amount of virus produced during an infection. This can be up to 10 mg virus per gram of leaf, representing up to 75% of the total nucleic acid content of the leaf. What are the effects of this vast amount of "Foreign" metabolism on the growth and nucleic acid metabolism of the leaf? Early investigations of RNA changes after TMV infection showed mainly slight increases in total RNA following infection (Basler and Commoner, 1956; Fry and Matthews, 1963; Röttger, 1965). Later investigations were concerned with the metabolism of specific RNAs, namely TMV-RNA and ribosomal RNA. Reddi (1963) concluded that microsomal (ribosomal) RNA was actively broken down during virus multiplication, and the products used for TMV-RNA synthesis. Later, Babos (1966) found no difference in the amounts of ribosomal RNA in healthy and TMV-infected leaves. Kubo and Tomaru (1968) reported a stimulation of uridine-^{3}H incorporation into 80 S ribosomes for a few days after inoculation.

I report here an investigation of RNA

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metabolism in healthy and TMV-infected tobacco leaves, using assay methods (Fraser, 1971) permitting quantitative changes in DNA, TMV-RNA, cytoplasmic ribosomal RNA (rRNA), chloroplast ribosomal RNA (crRNA), and transfer RNA (tRNA) to be followed during TMV multiplication. The growth and senescence of the healthy tobacco leaf are complex and involve extensive changes in contents of different RNA types. For a full understanding of the relationship between TMV-RNA and plant RNA metabolism, it was necessary to investigate the effects of virus multiplication on leaves of widely differing ages. Two TMV strains were used, *vulgare* (light green/dark green mosaic symptoms) and *flavum* (severe yellowing of leaf). These strains have different effects on the stability of chloroplast ribosomal RNA (Fraser, 1969). It was therefore of interest to compare the effects of the two strains on further aspects of leaf growth and RNA metabolism.

MATERIALS AND METHODS

Plants and inoculation. Tobacco plants (*Nicotiana tabacum* L. var. 'Samsun') were grown in rich garden soil in clay pots. The plants were kept in a greenhouse at temperatures of 20° night and 25–28° day.

Leaves 8, 13, 17, or 18 cm long on plants 20–25 cm tall were dusted with 500-mesh Carborundum and inoculated by rubbing with a glass spatula. The inocula were: control—sterile 0.067 *M* Na_2HPO_4–KH_2PO_4 buffer, pH 7.0; *vulgare*—0.1% purified TMV strain *vulgare* in phosphate buffer; *flavum*—TMV-strain *flavum*-infected leaves ground in 10 volumes of phosphate buffer. The leaves were washed with running tap water immediately after inoculation.

Primary, mechanical inoculation of leaves less than 5 cm long is unsatisfactory. Small leaves were secondarily infected by a method similar to those described by Zech (1952) and Nilsson-Tillgren *et al.* (1969). The first two fully expanded leaves on 20 cm tall plants (generally leaves 5 and 6 from the bottom) were primarily inoculated by rubbing as above. Two young leaves, of 1.5 and 0.5 cm length, were identified on each plant at the time of inoculation. Four days later, the leaves which had been 1.5 cm long were 5–7 cm long and showed vein-clearing symptoms over the whole surface. The leaves which had been 0.5 cm long at the time of primary inoculation had reached a length of 1.5 cm and developed mosaic symptoms with small islands of very dark green color. Comparable leaves on different plants began to exhibit these symptoms rather synchronously. Nilsson-Tillgren *et al.* (1969) showed that a high proportion of leaf cells is quickly infected by this method. It is of course difficult to state the exact time at which virus arrived in the young leaf. However, TMV-RNA could be detected by gel electrophoresis (Fraser, 1971) by the time the leaves which were 1.5 and 0.5 cm long at the time of primary inoculation had reached 5 and 1.5 cm, respectively. For this investigation, it was sufficient to know that these leaves were infected by the time they had reached this stage. The actual onset of infection, comparable to the time of rubbing of primarily infected leaves, must have been when the leaves were slightly smaller.

Nucleic acid determinations. At least three leaves were harvested at each time for each treatment. The midribs were removed. The leaves were quickly frozen on solid CO_2 and broken into small pieces. This allowed a random sampling of all regions of replicate leaves, and had no adverse effect on the quality or quantity of nucleic acids extracted. At least three 0.4-g samples of the frozen leaf mixture were separately processed for nucleic acid measurement for each treatment.

The extraction and assay of nucleic acids were performed as described in detail by Fraser (1971). The salient points of the method are: nucleic acids were extracted to 80% yield by a detergent/phenol method; purified by dialysis in the presence of sodium dodecyl sulfate and fractionated by electrophoresis on polyacrylamide gels (Loening, 1967). The gels were 6 mm diameter, 115 mm length; with a final acrylamide concentration of 2.2%, and were run for 2.5 hr at 67 V and 5 mA per gel. Amounts of DNA, TMV-RNA, rRNA, crRNA, and tRNA present were determined from the peak areas of these molecules in ultraviolet absorbance scans of the gels. Replicate extractions and

determinations of individual molecular types gave highly reproducible results.

RESULTS

Leaf Growth

The tobacco leaves used in these experiments grew, depending on season and position on the plant, to a maximum length of 14–20 cm (Fig. 1) and a maximum fresh weight of 3–5 g (Fig. 2). DNA accumulation (Fig. 3) in the healthy leaf stopped shortly after the leaf reached its maximum length, but fresh weight increase occurred for some time thereafter.

Infection of small leaves with TMV-inhibited growth, generally the earlier the infection, the greater the inhibition. Length increase was inhibited less than weight increase (Figs. 1A, B; 2A, B). Infection of the 1.5 cm-long leaf had an immediate inhibitory effect on length (Fig. 1A) and DNA (Fig. 3A) increase, although DNA increase in virus-infected leaves continued longer than in the control. The leaf infected when 5 cm long (Figs. 1B, 2B, 3B) continued to grow as well as the control and doubled its length before inhibition became apparent. The final length reached was slightly reduced by virus; the final weight and DNA content were reduced even more.

Infection of leaves which had reached their maximum length had no effect on their final weight (Fig. 2D) or DNA content (Fig. 3C). In particular there was no loss of DNA from the infected leaf, although DNA is lost from virus-infected leaves when they become necrotic. Necrosis did not occur within the times of these experiments. The small decline in DNA per leaf in all treatments in Fig. 3C occurs because DNA becomes more difficult to extract from aging leaves at the pH which is optimal for RNA extraction.

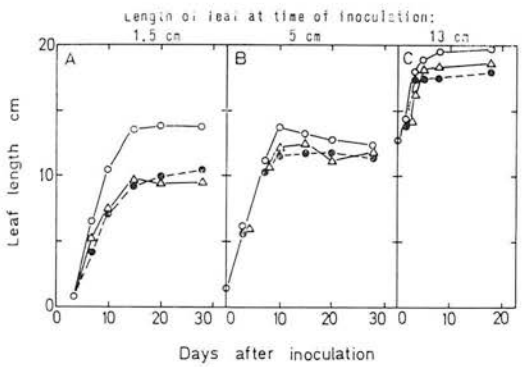

FIG. 1. Changes in leaf length with time after inoculation, for leaves of various sizes at time of inoculation. ○——○, Control; △——△, *vulgare*-infected; ●- - -●, *flavum*-infected. (A and B) Secondarily infected; (C) primarily inoculated.

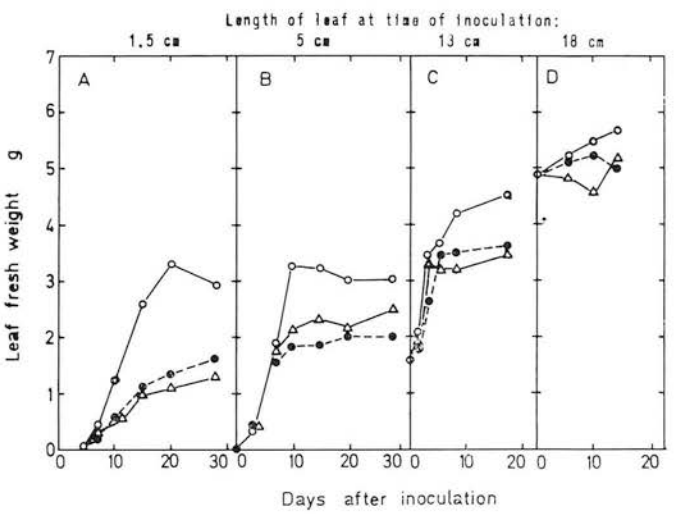

FIG. 2. Changes in leaf fresh weight with time after inoculation, for leaves of various sizes at the time of inoculation. ○——○, Control; △——△, *vulgare*-infected; ●- - -●, *flavum*-infected. (A and B) Secondarily infected; (C and D) primarily inoculated.

TMV-RNA

The duration and amount of TMV-RNA synthesis depended on the virus strain and the age of leaf inoculated. The duration of virus-RNA multiplication decreased with increasing length of leaf inoculated, from at least 25 days when 1.5 cm leaves were inoculated (Fig. 4A) to 5 days when old, full-length leaves were inoculated (Fig. 4D). Strain *flavum* had a slightly longer duration of multiplication than *vulgare* in old leaves, and considerably longer than *vulgare* when 5 cm leaves were infected.

Viral-RNA production per leaf was lower when very young or very old leaves were infected (Fig. 4,A and D). Assuming that the TMV-RNA was present in virus particles, the data may be expressed as milligrams of virus per leaf, taking TMV-RNA as 5% of total virus weight (Table 1). The highest virus concentrations (mg virus per gram of leaf) were reached in leaves infected when 1.5 to 8 cm long. Infection of leaves longer than 8 cm led to lower final virus concentrations. This effect was more pronounced with *flavum*: in young leaves it reached concentrations equal to those of

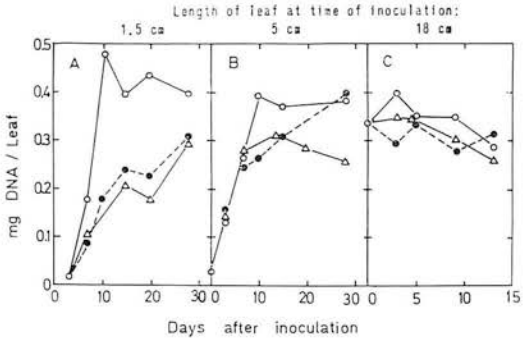

FIG. 3. Changes in amount of DNA per leaf with time after inoculation, for leaves of various sizes at the time of inoculation. ○——○, Control; △——△, *vulgare*-infected; ●- - -●, *flavum*-infected. (A and B) Secondarily infected; (C) primarily inoculated.

TABLE 1

MAXIMUM CONCENTRATIONS OF TMV STRAINS *flavum* AND *vulgare* FOUND WHEN LEAVES OF DIFFERENT SIZES WERE INOCULATED

Leaf length at time of inoculation (cm)	Maximum TMV-concentration reached in leaf (mg virus/g leaf)[a]	
	vulgare	*flavum*
1.5	6.67	6.30
5.0	8.10	7.10
8.0	6.26	—
13.0	4.95	—
17.0	3.78	1.78
18.0[b]	1.78	0.40

[a] Derived from 20 × the measured content of TMV RNA/g, assuming this RNA to be present in virus particles.

[b] Leaf inoculated 6 days after reaching 18 cm, its maximum length.

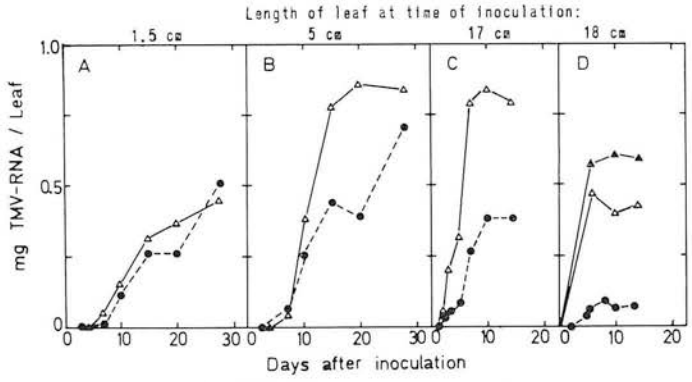

FIG. 4. Changes in the amount of TMV-RNA per leaf with time after inoculation, for leaves of various sizes at the time of inoculation. △——△, *vulgare*-infected; ●- - -●, *flavum*-infected. (A and B) Secondarily infected; (C) primarily inoculated; (D) primarily inoculated 6 days after reaching 18 cm, its maximum length. ▲——▲, Leaf excised and floated on water 1 day after primary inoculation with *vulgare*.

vulgare; in old leaves it multiplied very much less than *vulgare* (Table 1).

RNA

The ribosomal RNA content of the healthy leaf reached a peak of 1.0–1.5 mg per leaf when or slightly before the leaf reached its maximum length (Fig. 5, A, B). Virus infection of the 1.5 cm leaf led to an immediately reduced rate of rRNA accumulation and a very much reduced maximum rRNA content, at, however, the same time as in the control (Fig. 5A). Infection of a 5 cm leaf had no effect on the accumulation of rRNA (Fig. 5B), but 15 days after infection, the rate of loss of rRNA from the by then aging leaves became slower in virus-infected leaves. Leaves 35 days after infection with *vulgare* had twice the rRNA content of the controls; *flavum*-infected leaves had almost four times the control rRNA level.

Leaves infected at the peak of rRNA content (13 cm length, Fig. 5C) and during rRNA loss in aging (17 cm length, Fig. 5D) showed, similarly, a retardation of rRNA loss after infection. *Flavum*-infected leaves lost their rRNA more slowly than *vulgare*-infected leaves, which lost theirs more

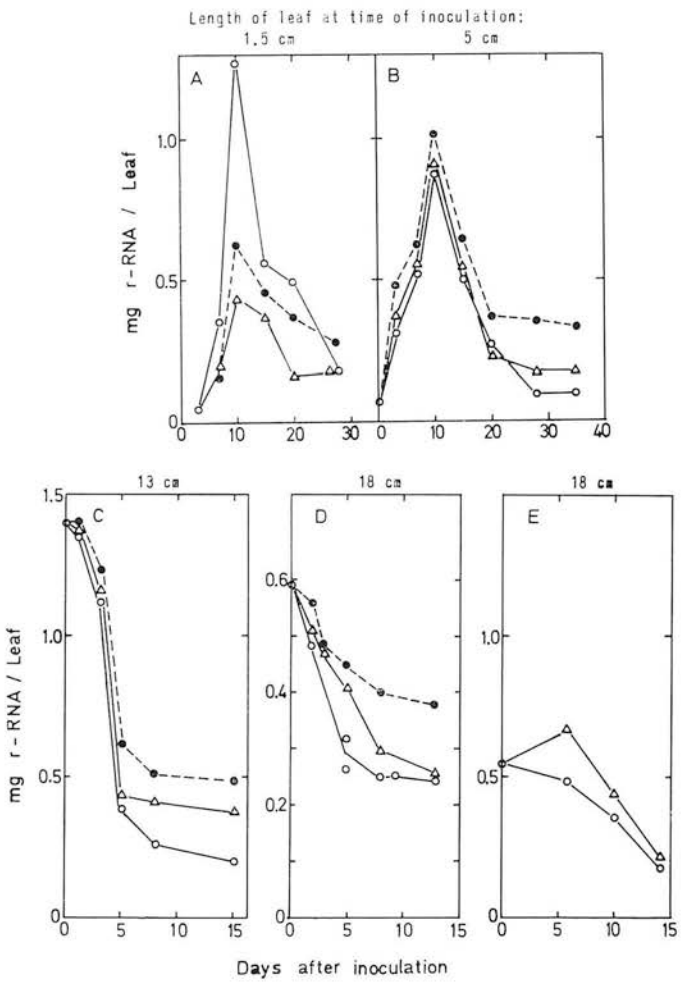

FIG. 5. Changes in the amount of ribosomal RNA per leaf with time after inoculation, for leaves of various sizes at the time of inoculation. ○——○, Control; △——△, *vulgare*-infected; ●– – –●, *flavum*-infected. (A and B) Secondarily infected; (C and D) primarily inoculated; (E) primarily inoculated 6 days after reaching 18 cm, its maximum length.

slowly than control leaves. With very old leaves, infected several days after reaching their maximum length (Fig. 5E), inoculation with *vulgare* actually caused a slight increase in rRNA content, although the net loss of rRNA resumed after 5 days.

Chloroplast ribosomal RNA in the healthy leaf reached its maximum concentration at the same time as cytoplasmic rRNA, but was lost very much more quickly as the leaf aged (Fig. 6A). I have reported in qualitative terms the effects of *flavum* and *vulgare* on chloroplast rRNA synthesis (see also Hirai and Wildman, 1969) and stability (Fraser, 1969). Similar results were reported for lettuce necrotic yellows virus-infected *Nicotiana glutinosa* by Randles and Coleman (1970). Here we consider two sets of quantitative data which confirm and extend these results. Infection of 1.5 cm leaves resulted in very low accumulations of chloroplast rRNA (Fig. 6A). The larger crRNA accumulation with *flavum* than with *vulgare* does not contradict the reportedly more severe cytopathic effects of *flavum* for crRNA stability (Fraser, 1969). The explanation lies in the mosaic form of the disease produced in these younger leaves. Dark-green, nonvirus-containing patches, which have high crRNA contents, were more frequent in *flavum*-infected leaves than in those infected with *vulgare*.

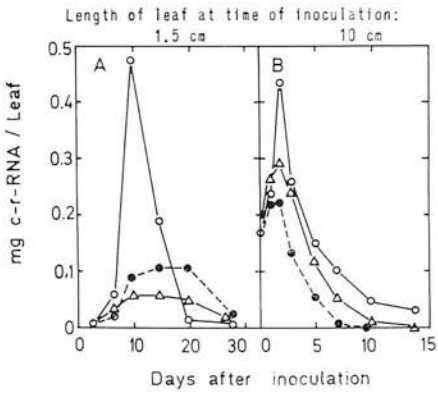

FIG. 6. Changes in chloroplast ribosomal RNA content per leaf with time after inoculation, for leaves of various sizes at the time of inoculation. ○——○, Control; △——△, *vulgare*-infected; ●- - -●, *flavum*-infected. (A) Secondarily infected; (B) primarily inoculated.

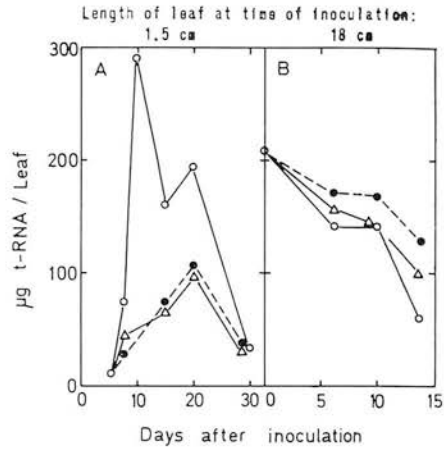

FIG. 7. Changes in the amount of transfer RNA per leaf with time after inoculation, for leaves of various sizes at the time of inoculation. ○——○, Control; △——△, *vulgare*-infected; ●- - -●, *flavum*-infected. (A) Secondarily infected; (B) primarily inoculated 6 days after reaching 18 cm, the maximum length.

Infection of the leaf with *flavum* or *vulgare* just before it reached its peak crRNA concentration (Fig. 6B) gave a rapid inhibition of crRNA accumulation. The subsequent loss of crRNA was fastest in *flavum*-infected leaves; *vulgare*-infected leaves behaved similar to the controls.

The effects of virus multiplication on tRNA content were broadly similar to those on rRNA content. Infection of young leaves by either virus strain (Fig. 7A) resulted in an inhibition of tRNA accumulation. When old leaves were infected (Fig. 7B), they lost tRNA at rates slower than in the control. As with rRNA, the rate of loss was lower in *flavum*-infected leaves than in *vulgare*-infected leaves.

DISCUSSION

Leaf Growth and Nucleic Acid Content

The life of the tobacco leaf falls into two periods. The first stage is one of rapid growth with increases in length, weight, DNA and all RNA types. The second phase is a period of senescence which occurs soon after the leaf reaches its maximum length and includes losses of RNAs of all types. Infection of the leaf by TMV inhibited the development of both stages. If the leaf

was infected when very young (1.5 cm), its growth (measured by length, weight, and nucleic acid increase) was inhibited. But by the time the leaf had reached about one-third of its final length (about 5 cm), many parameters of growth were resistant to inhibition by TMV infection. The only factor showing continued inhibition was fresh weight increase, a parameter which goes on increasing after all others measured in this study have reached their peaks (Figs. 2, B and C). Noteworthy when 5 cm leaves were infected was that rRNA and tRNA accumulations continued unimpaired, although considerable amounts of TMV-RNA were being synthesized at the same time. Only chloroplast rRNA synthesis showed a reduction in such circumstances.

Older leaves infected with TMV lost their rRNA and tRNA more slowly than did healthy leaves. This inhibition of the normal course of senescence by virus was more pronounced with strain *flavum* than with strain *vulgare*. Chloroplast rRNA again behaved in exactly opposite fashion to the other RNA types, being more rapidly degraded in the *flavum*-infected leaves. Results from ^{32}P incorporation studies (Fraser, in preparation) show that in older leaves there is a stimulation of cytoplasmic rRNA synthesis for a few days after inoculation, but during the main period of viral RNA accumulation the rRNA synthesis is very much reduced. Thus the higher contents of rRNA per leaf observed in infected older leaves (Fig. 5 C–E) are not only the results of an initially higher rate of synthesis; virus must also reduce the rate of degradation of existing rRNA.

By what mechanism does TMV exert these varied effects on host RNA metabolism? Some of the findings, such as inhibition of host rRNA accumulation in very young leaves, might be explained as a simple effect of competition for nucleotides between TMV and host RNA syntheses. However, the simultaneous but completely opposite effects of virus on chloroplast and cytoplasmic rRNA syntheses and stabilities (Figs. 5C and 6B) (Fraser, 1969) and the initial stimulation by virus of ^{32}P incorporation into rRNA (Fraser, in preparation) suggest that TMV may exert some directive control over host RNA metabolism. One facet of of this could be the blocking of RNA syntheses necessary for development, hence the observed inhibition of both growth and senescence phases. The TMV-sensitive synthetic activity which is necessary for the growth phase of development must take place when the leaf is very small: by the time the leaf has reached a length of 5 cm, it is competent to complete its growth phase even in the presence of TMV (Figs. 2B and 5B).

Whatever the mechanism of TMV interference with development, it will be noted that the situations produced have features favorable to virus multiplication, i.e., some reduction of competition by host RNA synthesis for nucleotides in very young leaves, and maintenance of the declining host ribosome population, necessary for viral protein synthesis, in infected older leaves.

In none of the situations investigated was any evidence found to support Reddi's (1963) contention that TMV infection and multiplication caused net degradation of existing cytoplasmic rRNA. However, examination of his experimental design shows that leaves were harvested for TMV and rRNA analyses at various times after infection, when they had reached a length of 10 cm. This is far short of the fully expanded size of tobacco leaves, and can only mean that the leaves harvested late in his experiments were infected when very small indeed. As shown here, infection of very young leaves with TMV inhibits the accumulation of rRNA (Fig. 5A), though such leaves can accumulate high concentrations of TMV-RNA (Fig. 4A). I suggest therefore that what Reddi measured was an inhibition of rRNA accumulation and by no means a virus-stimulated net degradation of rRNA.

The rRNA results from more mature leaves (Fig. 5, C–E) are broadly similar to those obtained by Babos (1966). The larger differences between control and infected leaves did not appear until periods exceeding the duration of his experiments.

Virus Multiplication

TMV-RNA was synthesized over longer periods and to higher concentrations when

younger leaves were infected (Fig. 4; Table 1). Strain *flavum* RNA was synthesized as much as *vulgare* RNA in younger leaves but much less than *vulgare* RNA in older leaves. These effects may be related to nucleotide availability. Comparison of Figs. 4 and 5 shows that the main virus RNA accumulation took place when host rRNA was declining. TMV-RNA synthesis in younger leaves, infected before they reached their maximum rRNA content, did not reach its full rate until rRNA decline had commenced. Further, the amount of viral RNA finally accumulated bore a very close relationship to the amount of rRNA degraded during leaf senescence. The lower synthesis of TMV-RNA in older leaves is probably a direct consequence of the smaller supply of nucleotides, because fewer ribosomes are left to be degraded.

Thus much of the nucleotide supply for TMV-RNA synthesis seems to come from the normal degradation of rRNA during leaf senescence. It must be stressed that TMV does not accelerate rRNA degradation; indeed the reverse is the case. It could be that the lower production of *flavum* RNA compared to *vulgare* RNA in older leaves (Fig. 4D) was because *flavum* is more effective in reducing the degradation of rRNA.

While in infected young leaves TMV-RNA synthesis did not become maximal until after the peak of rRNA content was reached, TMV-RNA synthesis did occur during the net rRNA accumulation phase. ^{32}P incorporation studies (Fraser, in preparation) show that much more ^{32}P was incorporated into rRNA than into TMV-RNA during an 8-hr incubation. Thus certainly during the rRNA accumulation phase, rRNA synthesis is a more active sink for nucleotides than TMV-RNA synthesis.

It may be assumed that the products of rRNA degradation in the old leaves of healthy plants are transported to the younger leaves to be utilized there. Virus RNA production in the old leaves is competing with the young leaves for nucleotides arising from old-leaf rRNA breakdown. It can be predicted that removing the competitive effect of the young leaves should increase the amount of viral RNA synthesis in old leaves. This is indeed the case. When old leaves were inoculated, excised, and floated on water, larger concentrations of virus were reached than in comparable leaves still on the plant (Fig. 4 D).

In the very youngest leaves infected the amount of TMV-RNA synthesized was in excess of the amount of rRNA degradation in these leaves. It is probable that viral RNA production in these leaves was considerably supported by the import of nucleotides arising from senescence in lower, older leaves. The equal production of *flavum* or *vulgare* RNAs in younger leaves could reflect the absence of any effect of *flavum* in preventing rRNA breakdown in the regions of production of the nucleotides. Uninoculated old leaves become systemically infected very slowly with virus from secondary sources.

ACKNOWLEDGMENTS

This work was supported by the Max-Planck-Gesellschaft zur Förderung der Wissenschaft. I thank Professor G. Melchers for encouragement, and Frl. Ute Klöpfer, Gerd Strobel, and Günther Scheer for capable technical assistance.

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J. gen. Virol. (1973), 18, 267–279
Printed in Great Britain

The Synthesis of Tobacco Mosaic Virus RNA and Ribosomal RNA in Tobacco Leaves

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(*Accepted* 17 *October* 1972)

SUMMARY

Young, maturing and aged tobacco leaves were infected with tobacco mosaic virus (TMV) strain *vulgare* or *flavum*. TMV-RNA synthesis and accumulation were followed. *Flavum* and *vulgare* RNAs had different patterns of synthesis and accumulation: *flavum*-RNA may be unstable.

In healthy leaves, ribosomal RNA synthesis (measured by [^{32}P] incorporation) increased to a peak before the leaf reached its maximum length then declined to 25% of the maximum as the leaf aged. TMV infection of a young leaf caused immediate and persistent inhibition of ribosomal RNA synthesis. Ribosomal RNA synthesis in older leaves showed three phases after TMV infection. (1) One day after inoculation, ribosomal RNA synthesis was higher than in healthy leaves. (2) During the main accumulation of TMV-RNA, ribosomal RNA synthesis was inhibited. (3) Following TMV-RNA accumulation, ribosomal RNA synthesis rose, often to levels higher than in healthy leaves. The half-life of ribosomal RNA in a TMV-infected leaf was found to be twice that in a healthy leaf.

These observations are discussed in relation to leaf development and virus multiplication.

INTRODUCTION

Tobacco mosaic virus (TMV) can multiply in a tobacco leaf until its RNA is 75% of the total nucleic acid present (Fraser, 1971). Certain aspects of host RNA metabolism are already known to be considerably altered after TMV infection. Chloroplast ribosomal RNA synthesis is inhibited (Fraser, 1969; Hirai & Wildman, 1969). In very young leaves, TMV infection inhibits aspects of growth, including accumulations of ribosomal RNA (r-RNA), transfer RNA and DNA. Infection of older leaves slows the loss of cytoplasmic r-RNA which occurs in the normal course of leaf senescence (Fraser, 1972).

The effects of TMV infection on cytoplasmic r-RNA synthesis have not previously been studied in detail. This paper reports an investigation of this topic. The multiplication patterns of two strains of TMV, *vulgare* and the more cytopathic *flavum*, and their effects on r-RNA synthesis were compared. These strains have different effects on other aspects of leaf RNA metabolism (Fraser, 1969, 1972). r-RNA synthesis was studied in leaves infected at different ages in the hope of understanding the effects of TMV on leaf development. An attempt was made to measure the rate of r-RNA turnover in healthy and infected leaves.

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METHODS

Plants and inoculation. Tobacco plants (*Nicotiana tabacum* L. var. 'Samsun') were grown in soil in clay pots. One week before inoculation, the plants were transferred to a climatic chamber and grown under continuous, mixed fluorescent and tungsten light of 7500 lx, at 25 ± 1 °C and 80 % relative humidity. Plants of similar height (20 to 25 cm) and appearance were selected. Leaves 10 or 17 cm long were primarily inoculated with TMV strains *vulgare* or *flavum* or with sterile phosphate buffer (control) and leaves 4 cm long were secondarily infected following primary inoculation of lower, expanded leaves (Nilsson-Tillgren, Kolehmainen-Sevéus & von Wettstein, 1969) as described previously (Fraser, 1972). The infected or sham-inoculated leaves were identified by light metal rings round the petiole. Equivalent leaves were used for the determination of RNA synthesis rates at various times after inoculation.

Radioactive incubation. Up to 30 days after infection, infected or sham-inoculated leaves were detached by cutting the petiole under water. The cut end was immersed in 0·5 ml water containing 0·5 mCi [^{32}P]-orthophosphate (Radiochemical Centre, Amersham). The entire volume of radioactive solution was taken up by the leaf within 1 h. For the remainder of the incubation the leaf was floated on sterile water.

Nucleic acid extraction. Nucleic acids were extracted from 0·4 g samples of leaf tissue by a detergents–phenol procedure (Fraser, 1971), and fractionated by electrophoresis on polyacrylamide gels (Loening, 1967). The gels were scanned for u.v. extinction at 265 nm with a Joyce Loebl 'Chromoscan', frozen in solid CO_2 and sliced transversely at 0·5 mm intervals. The slices were dried on a film base and radioactivity in each slice was counted in a Packard 460 gas-flow system.

Derivation of data. The following data were derived from the u.v. extinction and radioactivity scans of gels:

(1) The total incorporation of radioactivity into 25 S plus 18 S cytoplasmic r-RNA or into TMV-RNA was found by adding the radioactivities of the individual gel slices in the appropriate radioactivity peaks, and subtracting the general background of heterodisperse radioactivity on the gel, as shown in Fig. 1.

(2) The weight of DNA on the gel was calculated from the area of the DNA peak in the scan of u.v. extinction. The ratio peak area/weight of DNA was determined by electrophoresis of known amounts of pure *Escherichia coli* DNA. DNA migrates in polyacrylamide gels as a single, sharp peak, unless very highly sheared (Loening, 1967). The DNA content of a sample of leaf material was found by this method to be 130 ± 4 μg/g leaf (mean of eight determinations ± standard error). The DNA content was separately measured by the procedure of Schmidt & Thannhauser (1945) using the modified pre-washing procedure of Holdgate & Goodwin (1965). The value found was 144 ± 7 μg/g leaf. This confirms that the phenol-detergent extraction and gel peak-area method provides a reliable and reproducible estimate of leaf DNA content.

(3) The weight of TMV-RNA on the gel was calculated from its peak area on the u.v. extinction scan, as described by Fraser (1971). No allowance was made for double-stranded TMV-RNA, which probably has an electrophoretic mobility similar to DNA (Jackson *et al.* 1972). Published estimates of double-stranded TMV-RNA concentration in leaves (Shipp & Haselkorn, 1964; Babos, 1971) suggest that its concentration relative to that of single-stranded TMV-RNA is too low to introduce significant error into TMV-RNA or DNA measurements.

Method of presentation of results. [^{32}P] incorporation into r-RNA and TMV-RNA, and

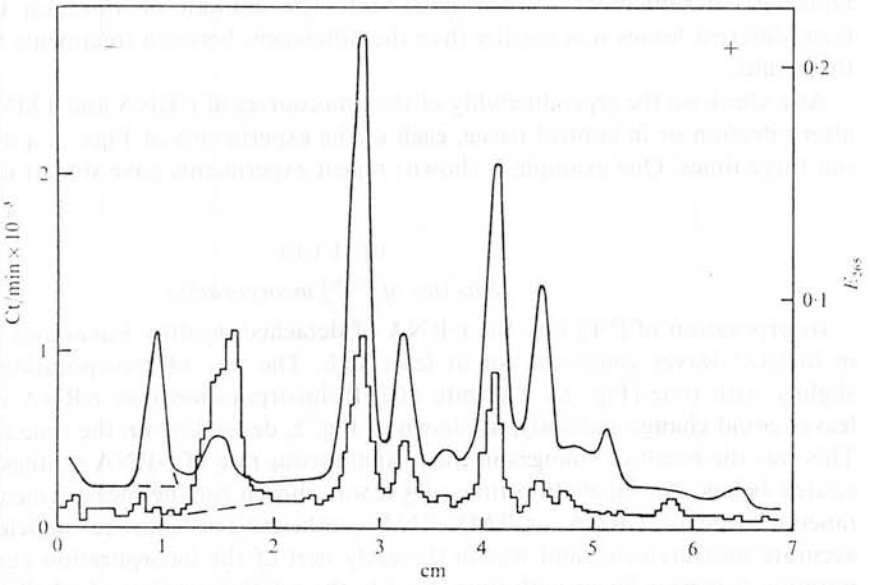

Fig. 1. Polyacrylamide gel electrophoresis of [^{32}P]-labelled nucleic acids extracted from a 13 cm long tobacco leaf two days after inoculation with TMV strain *vulgare*. The gel had an acrylamide concentration of 2·4 % and was run for 2·5 h at 5 mA, 8 V/cm gel length. The continuous curve shows extinction at 265 nm; the histogram radioactivity. The peaks are: at 1·0 cm, DNA; at 1·5 cm, TMV-RNA and at 2·8 and 4·2 cm, r-RNA. The broken line under the TMV-RNA radioactivity peak shows the delimitation of the peak for calculation of [^{32}P]-incorporation into TMV-RNA. The broken line under the DNA extinction peak shows how the peak area was obtained for calculation of the weight of DNA on the gel.

the amount of TMV-RNA have been expressed on a per μg DNA basis rather than on a per leaf or per μg r-RNA basis. This has several advantages. As corresponding RNA and DNA data were derived from the same gel, variation arising from differences in extraction and gel-loading was minimized. Incorporation of [^{32}P] into TMV-RNA and r-RNA could be directly compared. TMV infection certainly has drastic effects on leaf r-RNA contents (Fraser, 1972). If this is even partly an effect on degradation rather than synthesis, as this investigation suggests, then specific activity values for r-RNA ([^{32}P] incorporation into r-RNA/μg r-RNA) are meaningless as a guide to r-RNA synthesis.

TMV infection affects leaf DNA content only when very small leaves are infected. Changes in DNA content of leaves during growth are given in Fraser (1972) and closely parallel changes in leaf fresh weight, i.e. curves drawn on a per μg DNA basis are essentially similar to those drawn on a per g fresh weight basis.

Reproducibility of results. Estimates of [^{32}P] incorporation by the gel method are likely to be free from errors arising from [^{32}P]-containing contaminants, as a clean, definite peak is measured. The RNA extraction procedure has been shown to give an 80 % yield; reproducibility of extraction is extremely high (Fraser, 1971). A control experiment showed that measurements of [^{32}P] incorporation by this method are also highly reproducible. Eight identical leaves were incubated with [^{32}P] 5 days after inoculation with TMV. [^{32}P] incorporation into r-RNA was 5040 ± 356 ct/min/μg DNA (mean $\pm$ standard error) from eight samples taken from a single leaf frozen on solid CO_2 and crushed finely before sampling, and 4807 ± 678 ct/min/μg DNA from eight samples taken from eight different leaves. The standard errors show that the variation between leaves was larger than between

replicate determinations on the same leaf. The amount of variation between samples from different leaves was smaller than the differences between treatments to be reported in the results.

As a check on the reproducibility of the timecourses of r-RNA and TMV-RNA syntheses after infection or in control tissue, each of the experiments of Figs. 3, 4 and 5 was carried out three times. One example is shown; repeat experiments gave similar curves.

RESULTS

Kinetics of [^{32}P] *incorporation*

Incorporation of [^{32}P] into the r-RNA of detached, healthy leaves and into TMV-RNA in infected leaves continued for at least 50 h. The rate of incorporation decreased only slightly with time (Fig. 2). The rate of [^{32}P] incorporation into r-RNA in TMV-infected leaves could change suddenly, as shown in Fig. 2, depending on the time after inoculation. This was the result of changes in the instantaneous rate of r-RNA synthesis, to be demonstrated below. An incubation time of 5 h was chosen for the measurement of the 'instantaneous' rate of r-RNA or TMV-RNA synthesis. This allowed sufficient labelling for accurate measurement, and was in the early part of the incorporation curve where incorporation was near linear with time. By 5 h, the relative amount of labelling of the r-RNA precursor (Rogers, Loening & Fraser, 1970) was too small to cause significant errors in estimates of [^{32}P] incorporation into TMV-RNA, which has a similar electrophoretic mobility.

Synthesis of TMV-RNA

Young leaves were secondarily infected with TMV strain *vulgare* (Nilsson-Tillgren *et al.* 1969). The first sign of virus in the young leaves was [^{32}P] incorporation into TMV-RNA, detectable 3 days after primary inoculation of the lower leaves (Fig. 3). By this time the leaf was 4 cm long. The synthesis of TMV-RNA was at a maximum between 3 and 6 days after the first detection of virus RNA synthesis, then declined slowly until after 25 days no pulse-labelling whatsoever of TMV-RNA could be detected. The concentration of TMV-RNA in the leaf rose rapidly during virus RNA synthesis and became constant after synthesis declined, at a level of 0·5 mg TMV-RNA/g fresh weight of leaf.

The leaves primarily inoculated when 10 cm long grew to a maximum length of 17 cm. In *vulgare*-infected leaves, TMV-RNA synthesis was detectable by [^{32}P] incorporation 1 day after inoculation, was at a maximum rate 3 days after inoculation then gradually declined (Fig. 4). The TMV-RNA content of the leaf increased in a sigmoid fashion and became constant after 10 days.

In leaves inoculated with strain *vulgare* after they had reached their maximum length of 17 cm, *vulgare* RNA synthesis and accumulation followed timecourses similar to those observed when 10 cm leaves were inoculated, except that the initial lag in TMV-RNA synthesis was shorter and the final amount of TMV-RNA was less and was reached earlier (Fig. 5).

The multiplication of TMV strain *flavum* when 10 or 17 cm leaves were infected was rather different from the *vulgare* multiplication pattern. *Flavum* replicated to reach an RNA concentration only about 40 % of that reached by *vulgare* (Figs. 4, 5). The accumulation of *flavum*-RNA lasted if anything only slightly longer than *vulgare*-RNA accumulation. [^{32}P]-incorporation curves show that the labelling of *flavum*-RNA tended to build up more slowly than *vulgare*-RNA labelling, but that *flavum*-RNA labelling remained at a high

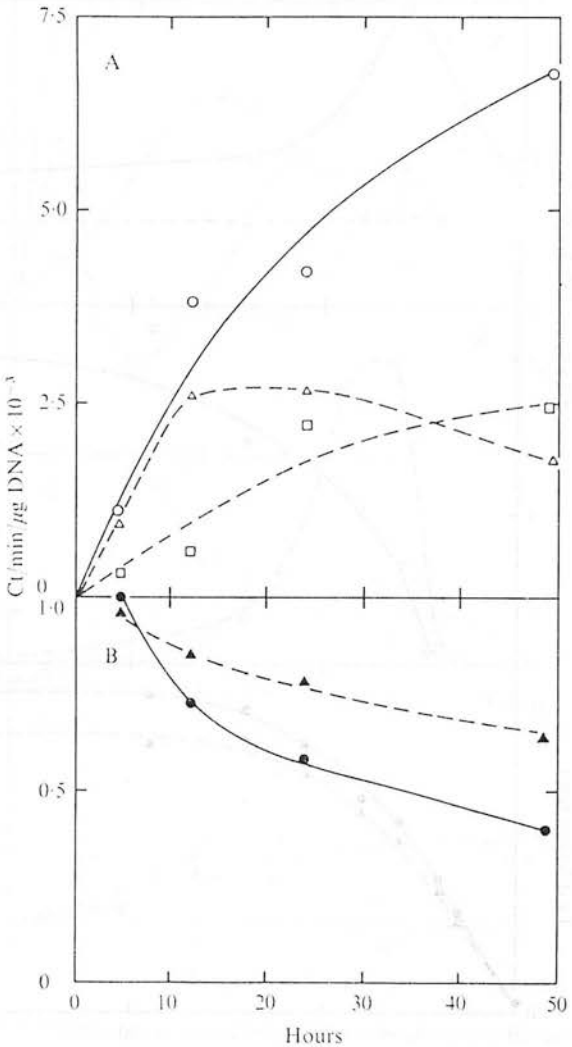

Fig. 2. Kinetics of [^{32}P]-orthophosphate incorporation into r-RNA and TMV-RNA in healthy and TMV strain *vulgare* infected leaves. Two days before the [^{32}P] incubation, leaves 13 cm long were inoculated with *vulgare* or sterile phosphate buffer; 4·5 h after the start of the [^{32}P] incubation, samples or control and TMV-infected leaves were cut into strips 2 × 10 mm and infiltrated under vacuum with either sterile water (A) or 50 μg/ml actinomycin D (B). The leaf strips were floated on water or actinomycin D solution in Petri dishes, under continuous illumination.

(A) ○—○, [^{32}P] incorporation into r-RNA of healthy leaf; △--△, [^{32}P] incorporation into r-RNA or TMV-infected leaf; □--□, [^{32}P] incorporation into TMV-RNA.

(B) After infiltration with actinomycin D at 4·5 h. ●—●, [^{32}P] in r-RNA of healthy leaf; ▲--▲, [^{32}P] in r-RNA of TMV-infected leaf.

level long after the rate of *vulgare*-RNA synthesis had dropped, and after a steady *flavum*-RNA level in the leaf had been reached.

r-RNA synthesis

In the healthy tobacco leaf, the rate of r-RNA synthesis rose to a maximum when the leaf had reached between 60 and 80 % of its maximum length (Figs. 3, 4). This is slightly before

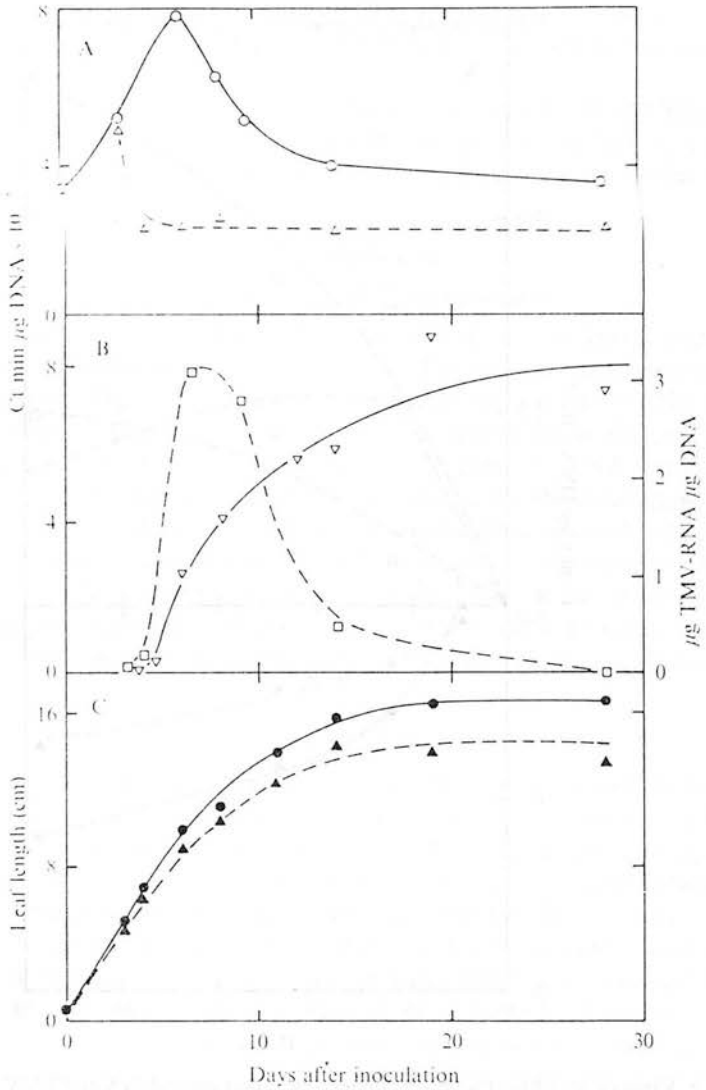

Fig. 3. r-RNA and TMV-RNA synthesis after secondary infection of small leaves by TMV strain *vulgare*.

(A) [^{32}P] incorporation during a 5 h incubation into r-RNA of healthy (○—○) and TMV-infected leaves (△– -△).

(B) □—□, [^{32}P] incorporation into TMV-RNA; ▽—▽, μg TMV-RNA/μg DNA.

(C) Changes in leaf length with time after inoculation: ●—●, healthy leaf; ▲– -▲, TMV-infected leaf.

the peak in leaf r-RNA content, which occurs at about the time when maximum length is reached (Fraser, 1972). The rate of r-RNA synthesis in the healthy leaf declined as the leaf aged. The low, fairly stable rate of [^{32}P] incorporation into r-RNA in aged leaves (Fig. 5) was about one quarter of the maximum rate recorded in young leaves (Fig. 4).

When a 4 cm long leaf was infected by TMV strain *vulgare* (Fig. 3), r-RNA synthesis began to decline as soon as TMV-RNA synthesis became detectable, then stayed at a constant, low rate. r-RNA synthesis did not rise after the end of TMV-RNA synthesis.

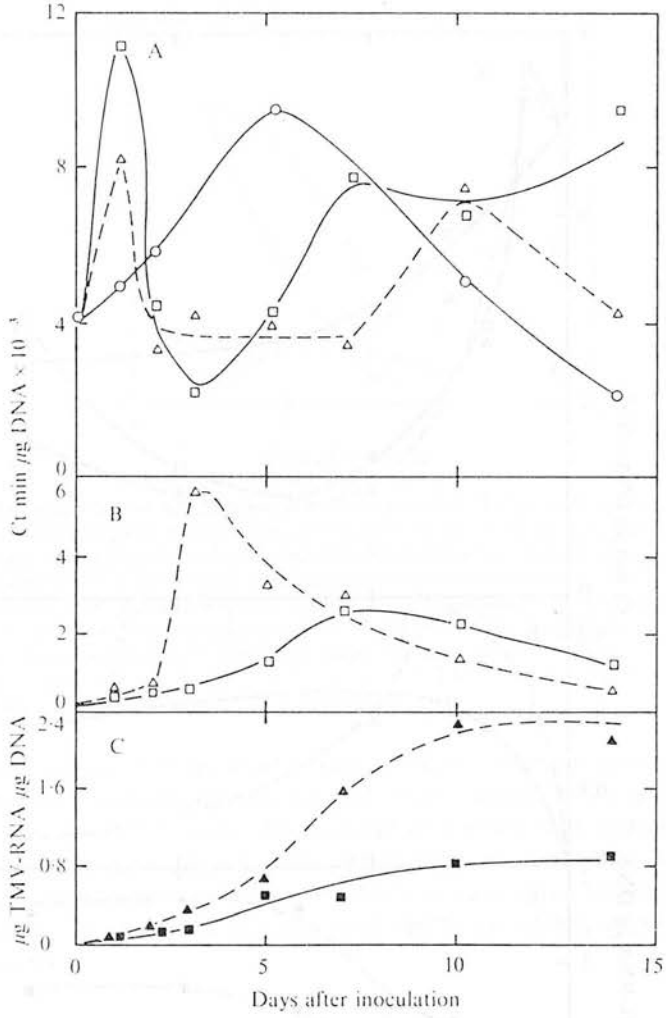

Fig. 4. r-RNA and TMV-RNA synthesis after primary inoculation of 10 cm long leaves.
(A) [^{32}P] incorporation during a 5 h incubation into r-RNA of healthy (○—○), TMV strain *vulgare*-infected (△- -△) and TMV strain *flavum*-infected leaves (□—□).
(B) [^{32}P] incorporation into TMV-RNA: △- -△, *vulgare*; □- -□, *flavum*.
(C) Changes in leaf content (μg/μg DNA) of TMV-RNA: ▲- -▲, *vulgare*; ■—■, *flavum*.

When 10 or 17 cm long leaves were infected with virus (Figs. 4, 5), the subsequent pattern of r-RNA synthesis was very different from that in healthy leaves. In the first day after inoculation, [^{32}P] incorporation into r-RNA in virus-infected leaves rose to a level higher than in control leaves. The post-inoculation stimulation of r-RNA synthesis in older leaves was not a result of rubbing the leaf during primary inoculation. The control leaves were sham-inoculated and showed no stimulation of r-RNA synthesis. A comparison between two healthy leaves, one sham-inoculated 1 day previously and one not, showed no significant difference in the rate of [^{32}P] incorporation into r-RNA.

Following the initial stimulation, r-RNA synthesis in infected leaves dropped to a level below that in control leaves. This reduced rate of r-RNA synthesis coincided with the main period of accumulation of TMV-RNA. Finally, after the end of TMV-RNA accumulation,

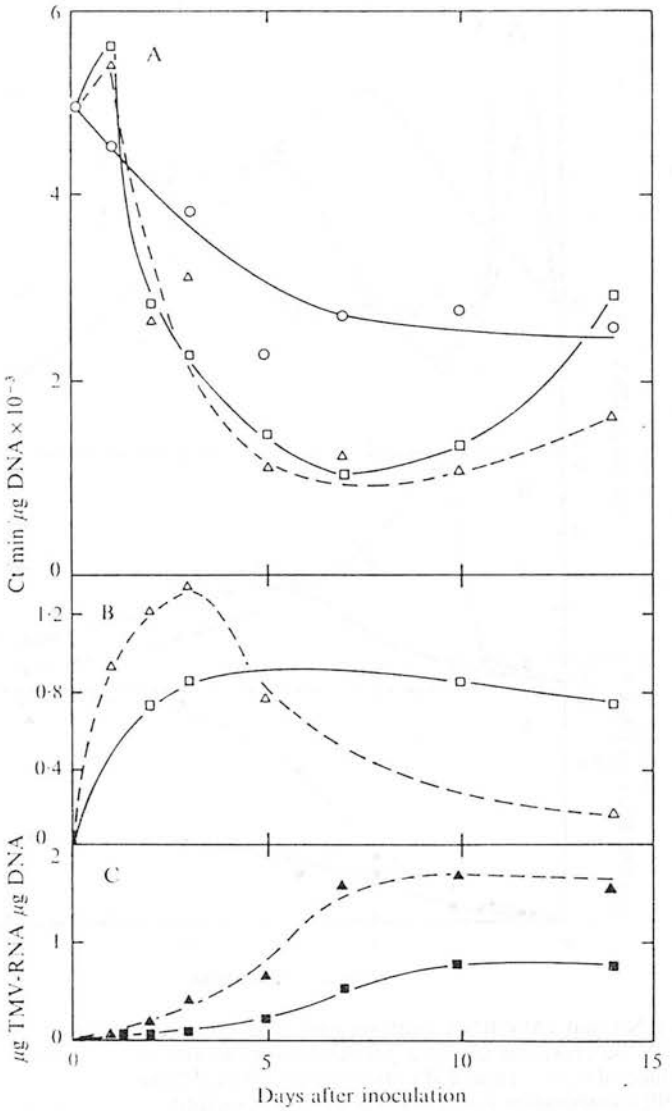

Fig. 5. r-RNA and TMV-RNA synthesis after primary inoculation of 17 cm long tobacco leaves.
(A) [^{32}P] incorporation during a 5 h incubation into r-RNA of healthy (○—○), TMV strain *vulgare*-infected (△--△) and TMV strain *flavum*-infected leaves (□—□).
(B) [^{32}P] incorporation into TMV-RNA: △--△, *vulgare*; □—□, *flavum*.
(C) Changes in leaf content (μg/μg DNA) of TMV-RNA: ▲--▲, *vulgare*; ■—■, *flavum*.

the rate of r-RNA synthesis in infected leaves again rose. In leaves inoculated when 10 cm long, the rate of r-RNA synthesis after the end of TMV-RNA synthesis was higher than in control leaves for some days. This was especially true of leaves infected with TMV strain *flavum*. The late increase in r-RNA synthesis in leaves inoculated when 17 cm long was also more pronounced with *flavum* than with *vulgare*, but for both strains was less than the increase in leaves inoculated when 10 cm long.

Fig. 6 shows the results of an experiment which examined the effects on r-RNA synthesis of TMV multiplication in young leaves which had been artificially forced into a state

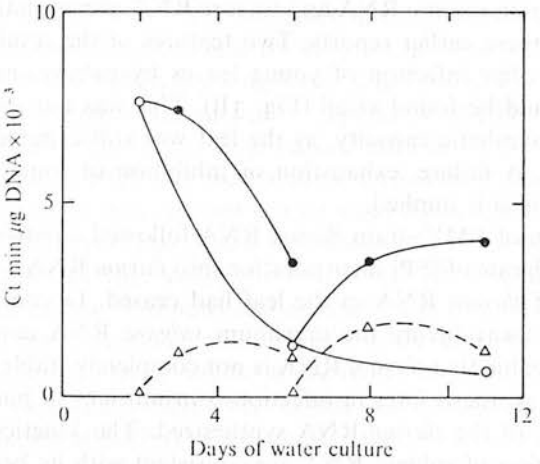

Fig. 6. r-RNA and TMV-RNA labelling in tobacco plantlets. Tobacco plantlets were grown on acid-washed sand with complete mineral nutrient solution. When the longest leaf was 4 to 5 cm long, the roots were washed free from sand and placed in deionized water. Leaves more than 1 cm long were inoculated with TMV strain *vulgare* 2 or 6 days after transfer to water culture. Control plants were sham-inoculated with sterile phosphate buffer. For [^{32}P] incubations, plantlets were placed with their roots in 0·5 mCi [^{32}P]-orthophosphate in 0·3 ml water. This was taken up within 90 min. The plants were then supplied with deionized water for the remainder of the 5 h incubation. [^{32}P] incorporation into r-RNA in healthy (○—○) and TMV-infected plants (●—●), and [^{32}P] incorporation into TMV-RNA (△--△) were determined.

similar to senescence in older leaves. The rate of r-RNA synthesis in young tobacco plantlets grown in sand with mineral-nutrient solution drops rapidly when the plantlets are transferred to deionized water culture. The content of r-RNA falls. Infection of the plantlets with TMV-*vulgare* 2 days after transfer, while the decline in r-RNA synthesis rate was rapid, slowed the decline. Infection with TMV 6 days after transfer, when the rate of r-RNA synthesis had stabilized at a low level, led to an r-RNA synthesis rate three times the control value.

r-RNA turnover

I tried to measure directly the effects of TMV infection on the rate of r-RNA degradation *in vivo*. Leaf tissue was labelled with [^{32}P] then infiltrated with actinomycin D at a concentration of 50 μg/ml, which is sufficient to stop all further r-RNA synthesis. Changes in the radioactivity of r-RNA after administration of actinomycin D then represent a degradation timecourse. Fig. 1 shows that radioactivity was lost more slowly from r-RNA in TMV-infected leaves than in control leaves. The half-life of r-RNA in healthy leaves, under these experimental conditions, was 40 h, in TMV-infected leaves about 80 h.

DISCUSSION

TMV-RNA synthesis

The kinetics of TMV multiplication are now well documented. Commoner *et al.* (1950) recorded the curve of increase of TMV coat-protein. Kubo & Tomaru (1968) reported changes in the incorporation of [^{3}H]-uridine and [^{14}C]-valine into TMV and changes in infectivity after infection. Fraser (1972) showed the pattern of TMV-RNA accumulation and how this varies with virus strain and the age of leaf infected. The data for incorporation

of [^{32}P] into TMV strain *vulgare* RNA and *vulgare* RNA accumulation shown in Figs. 3 to 5 are consistent with these earlier reports. Two features of the results, however, are new.

Twenty-five days after infection of young leaves by *vulgare*, no incorporation of [^{32}P] into TMV-RNA could be found at all (Fig. 3B). This was not merely a result of general debilitation of leaf synthetic capacity, as the leaf was still capable of r-RNA synthesis at this time (Fig. 3A). A failure, exhaustion or inhibition of some part of the TMV-RNA synthesizing mechanism is implied.

The multiplication of TMV strain *flavum* RNA followed a pattern different from that of *vulgare* RNA. A high rate of [^{32}P] incorporation into *flavum* RNA was maintained even after net accumulation of *flavum* RNA in the leaf had ceased. In contrast, the rate of *vulgare* RNA synthesis fell away before the maximum *vulgare* RNA concentration was reached (Figs. 4, 5). It is possible that *flavum* RNA is not completely stable, and turns over, or that *flavum* coat-protein synthesis later in infection is insufficient to build degradation-resistent virus particles with all the *flavum*-RNA synthesized. The kinetics of [^{32}P] incorporation into, and accumulation of *vulgare* RNA are consistent with its being highly stable.

r-RNA synthesis immediately after infection

In the first 1 or 2 days after infection, r-RNA synthesis was inhibited in leaves infected when 4 cm long; vastly stimulated in leaves infected when 10 cm long and slightly stimulated in leaves infected when 17 cm long (Figs. 3 to 5). When young leaves were artificially forced into a senescent phase, a stimulation of r-RNA synthesis followed infection (Fig. 6). Kubo (1966) reported a stimulation of RNA synthesis shortly after infection with TMV. Kubo & Tomaru (1968) found no stimulation of [^{3}H]-uridine incorporation into 80 S ribosomes after TMV infection of young leaves, but a stimulation of incorporation in older leaves. There is therefore a fundamental difference in the response of young, and older or senescent tissues to TMV infection.

TMV infection can certainly block leaf growth, measured by increases in length, weight, DNA, r-RNA and t-RNA, if the leaf is infected while very young, e.g. about 1·5 cm long. By the time the leaf reaches a length of 5 cm, its further growth, measured by all these parameters, is substantially resistant to inhibition by TMV (Fraser, 1972). Leaves infected when 4 cm long (Fig. 3) were intermediate between these two cases. Certain parameters of growth, such as length increase (Fig. 3C) and DNA increase (not shown) were but marginally inhibited by TMV infection; the increasing rate of r-RNA synthesis which is a part of leaf growth could still be inhibited. It is remarkable that the infected leaves were able to treble their length, and to grow almost as well as healthy leaves, despite severely curtailed r-RNA synthesis.

The amount of early stimulation of r-RNA synthesis after infection of older leaves declined with the age of the leaf. This was probably a result of the inability of older, senescent leaves to support vastly increased r-RNA synthesis. The relatively small amount of TMV-RNA synthesized (Fig. 5) is another guide to the impaired synthetic capacity of very old leaves.

r-RNA synthesis during TMV-RNA accumulation

During the major TMV-RNA accumulation in the leaf, r-RNA synthesis in all ages of leaf infected was depressed to less than one half of the control leaf level (Figs. 3 to 5). At the time of maximum TMV-RNA synthesis, leaves infected with *vulgare* when 4 cm long showed a level of [^{32}P] incorporation into TMV-RNA four times the [^{32}P] incorporation into r-RNA, a convincing demonstration of how much the RNA metabolism of the young leaf is turned over to virus RNA synthesis. Corresponding values for *vulgare* RNA labelling:r-RNA

labelling in older leaves were 1·4:1 in leaves infected when 10 cm long and 0·6:1 when 17 cm long leaves were infected. These results are consistent with the final virus concentrations reached, which are highest in young leaves (Figs. 3 to 5; Fraser, 1972). Kubo & Tomaru (1968) reported a decrease in the rate of incorporation of [^{3}H]-uridine into 80 S ribosomes at this time after infection. Pring (1971) also found in barley strip mosaic virus-infected barley leaves that more [^{32}P] was incorporated into virus RNA than into host RNA, and that host RNA synthesis was reduced during virus RNA synthesis.

It is tempting to try to explain the lowered rate of r-RNA synthesis during TMV-RNA synthesis as a result of the competition by TMV-RNA synthesis for nucleotides. But comparison of the rates of synthesis and accumulation of *flavum* and *vulgare* RNAs (Figs. 4, 5) suggests that the competitive inhibition of r-RNA synthesis from *flavum* RNA synthesis should be much less than from *vulgare* RNA synthesis. Actually the inhibitions of r-RNA synthesis by the two strains were very similar, suggesting that there may be some non-competitive inhibition of r-RNA synthesis. One case is already known of an inhibition of a specific RNA synthesis by non-competitive means after TMV infection. Just after infection, while cytoplasmic r-RNA synthesis is unimpaired or stimulated, chloroplast r-RNA synthesis is completely inhibited (Fraser, 1969; Hirai & Wildman, 1969).

The considerable TMV-RNA synthesis during this period makes it unlikely that the reduction of [^{32}P] incorporation into r-RNA was caused by some effect of infection on nucleotide pool size or rate of incorporation of inorganic phosphate into nucleotides.

r-RNA synthesis following TMV-RNA accumulation

r-RNA synthesis after the major TMV-RNA accumulation remained inhibited in leaves infected when 4 cm long and rose in leaves older when infected. Clearly the amount of stimulation or inhibition of r-RNA synthesis after the end of TMV-RNA synthesis is similar to the degree of stimulation or inhibition of r-RNA synthesis immediately after infection in each of the three ages of leaf infected. Taking the universal depression of r-RNA synthesis during TMV-RNA accumulation as a result of separate factors, possibly competitive, there are two ways of looking at r-RNA synthesis after TMV-RNA synthesis has declined. The effect of TMV on r-RNA synthesis seen immediately after infection may be a long-standing one, which reappears after the separate inhibition by multiplying TMV-RNA has lessened. The alternative explanation is based on an effect of TMV infection on leaf development. The losses of r-RNA and t-RNA content which are features of normal leaf senescence are retarded in TMV-infected older leaves, i.e. TMV blocks the senescent phase of development, just as it blocks the growth phase in infected young leaves (Fraser, 1972). The falling rate of r-RNA synthesis is also a facet of leaf senescence (Figs. 3 to 5). A blocking of this by TMV would lead to a rate of r-RNA synthesis higher than in control leaves after the ending of the separate inhibition of r-RNA synthesis during TMV-RNA accumulation. This is what occurs (Figs. 4, 5). It is consistent with this theory that *flavum* was always found to give a greater rise in late r-RNA synthesis than *vulgare*, as *flavum* also caused greater retardation than *vulgare* of the losses of r-RNA content during ageing (Fraser, 1972).

In TMV-infected, cultured tomato root-tips (R. S. S. Fraser, U. Klöpfer & D. A. Greenberg, in preparation) we have found a similar stimulation of r-RNA synthesis after the ending of TMV-RNA synthesis. In the healthy root, r-RNA synthesis declines with distance from the tip. TMV-RNA synthesis is localized behind the tip. Cells further from the tip, in which TMV-RNA synthesis has slowed, have r-RNA synthesis rates much higher than in healthy root-tips.

r-RNA turnover

The retardation by TMV of the loss of r-RNA content during leaf ageing could be caused by a stimulation of r-RNA synthesis or an inhibition of *in vivo* degradation. Considering the varied but often inhibitory effects of TMV on r-RNA synthesis (Figs. 4, 5) it seems unlikely that the maintenance of higher r-RNA content in older leaves is significantly due to increased r-RNA synthesis. The results of Fig. 1 suggest that r-RNA content is maintained because TMV infection slows the rate of *in vivo* degradation of r-RNA. This conclusion assumes that actinomycin D had no effect on r-RNA degradation and that r-RNA degradation was unaffected by the complete cessation of r-RNA synthesis.

Ribosomes and TMV multiplication

Infection by TMV forces the leaf to synthesize a vast amount of virus coat-protein, which requires ribosomes. TMV has very varied effects on r-RNA metabolism, but most of these result in situations favourable for virus multiplication. The inhibition of r-RNA synthesis during TMV-RNA accumulation reduces the competition for nucleotide substrates. Several effects combine to ensure that a high ribosome concentration is created for TMV protein synthesis: r-RNA synthesis is stimulated before and after TMV-RNA synthesis in older leaves; the normal loss of ribosomes during senescence is retarded. The permanent and severe inhibition of r-RNA synthesis when young leaves are infected can be of little significance for coat-protein synthesis, as the actual ribosome concentration in young leaves is much higher than in older leaves.

It is not known how TMV stimulates or inhibits r-RNA synthesis: whether the influence is through intermediary metabolism or if there is a direct effect on gene activity. The effects of TMV on growth and senescence of leaves open up the possibility of TMV influencing messenger RNA synthesis involved in the control of leaf development. An indication of the degree of sophistication of the control by TMV over leaf RNA metabolism is provided by the following simultaneous effects of *flavum*: stimulation of cytoplasmic r-RNA synthesis (Fig. 4); retardation of *in vivo* degradation of r-RNA (Fraser, 1972); inhibition of chloroplast r-RNA synthesis and acceleration of *in vivo* degradation of chloroplast r-RNA (Fraser, 1969).

This work was supported by the Max-Planck-Gesellschaft zur Förderung der Wissenschaft. I thank Professor G. Melchers for facilities and encouragement and Frl. Ute Klöpfer for excellent technical assistance.

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(*Received* 14 *August* 1972)

Reprinted from VIROLOGY, Volume 52, No. 1, March 1973

VIROLOGY 52, 275–280 (1973)

The Synthesis of TMV–RNA and Ribosomal RNA in Cultured Tomato Root Tips

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Accepted December 12, 1972

Cultured tomato root tips were infected with tobacco mosaic virus (TMV). The synthesis of TMV-RNA and ribosomal RNA was measured in healthy and TMV-infected roots by ^{32}P labeling and polyacrylamide gel electrophoresis. The region of most active TMV-RNA synthesis was 20–60 mm behind the tip. TMV-RNA was just over 4% of the total nucleic acid present in the entire infected root culture. Incorporation of ^{32}P into ribosomal RNA was greatly stimulated by TMV infection. This is discussed in relation to the increased size of the ^{32}P pool in roots infected by TMV: it is concluded that TMV infection stimulates ribosomal RNA synthesis. The region of the infected root showing high stimulation of ribosomal RNA synthesis coincided with the area of high TMV-RNA synthesis.

INTRODUCTION

Cultured tomato root tips may be infected with tobacco mosaic virus (TMV) (White, 1934; Bergmann, 1958). Studies by infectivity assay of the distribution of viruses in root tips have suggested that the apical meristem is uninfected (Crowley and Hanson, 1960; Smith and Schlegel, 1964). We have studied the distribution of TMV–RNA synthesis along the length of the root tip, and have shown differences in the rates of ribosomal RNA (rRNA) synthesis along healthy and infected root tips.

MATERIALS AND METHODS

Roots and Inoculation

Tomato roots were cultured in White's (1934) medium and infected with TMV strain *vulgare* as described by Bergmann (1958). All cultures used were derived from a single tomato seedling. Subculturing was performed at intervals of 2–3 weeks. The inoculum for a subculture was the apical 40 mm of a lateral root on the parent root culture. This inoculum grew to be about 200 mm long in 2–3 weeks, developed lateral roots, and in turn served as parent root for further subcultures. RNA synthesis was examined in roots 60 and 200 mm long, two passages after infection of the culture with TMV or sham inoculation of control roots. The maintenance of high levels of infectivity in TMV-infected root tip lines (Bergmann, 1959) was checked by infectivity assay on leaves of 'Xanthi-nc' tobacco.

^{32}P Incubation and RNA Extraction

Individual root tips were placed on a glass plate and covered with a thin film of White's medium lacking phosphate but containing 1.3 mCi [^{32}P]orthophosphate per ml (Radiochemical Centre, Amersham, U. K.; sp act 84 Ci per mg P). After 70 min at 24°C, the roots were returned to their original culture medium, containing 0.14 m*M* phosphate, for a 6-hr "chase" incubation. The roots were then chilled and cut transversely into segments 5–60 mm long. Lateral roots were discarded. Each segment was homogenized in 20 vol of Tris–HCl 50 m*M*; pH 7.8 at 0°C; sucrose 0.3 *M*; KCl 50 m*M*; $Mg(CH_3COO)_2$

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1 mM; dithiothreitol 5 mM in an Eppendorf 1.5 ml conical centrifuge tube fitted with a tapering Teflon pestle motor driven at 200 rpm. With this apparatus as little as 20 μl could be homogenized. Nucleic acids were extracted from the homogenate by a detergents–phenol procedure which gives a high, reproducible yield (Fraser, 1971).

The total pool of ^{32}P in each root segment was estimated roughly by counting aliquots of the supernatant fraction remaining after alcohol precipitation of the extracted nucleic acids.

RNA was fractionated on 2.1% polyacrylamide gels (Loening, 1967) for 3.5 hr at 5 mA per gel, 7 V per cm gel length. The gels were scanned at 265 nm in a Joyce–Loebl "Chromoscan" for the distribution of uv-absorbing nucleic acid peaks. After freezing in solid CO_2, the gels were sliced transversely at 0.5-mm intervals, the slices were dried, and the radioactivity in each slice was measured in a Packard 460 gas-flow system. Typical uv absorbance and radioactivity scans of gels are shown in Fig. 1. The total incorporation of ^{32}P into TMV–RNA or rRNA was calculated from the sum of the radioactivities of the individual gel slices composing the radioactivity peaks for TMV–RNA or the two ribosomal RNAs, allowing for the background of polydisperse labeling on the gel.

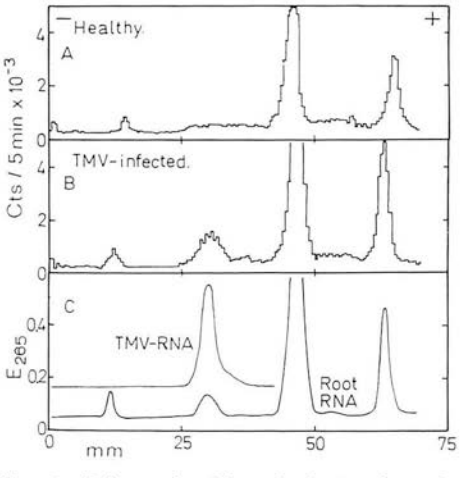

FIG. 1. Polyacrylamide gel electrophoresis of nucleic acids from tomato root tip cultures. A. ^{32}P-labeled nucleic acids from a healthy root. B. ^{32}P-labeled nucleic acids from a TMV-infected root. C. Ultraviolet absorbance scans of gels of nucleic acids from a TMV-infected root and from purified TMV particles. The peaks are: at 12–15 mm, DNA; at 30 mm, TMV–RNA, and at 45 and 63 mm, ribosomal RNA. Electrophoresis was from left to right.

RESULTS

Healthy and virus-infected roots grew in length at the same rate; this agrees with the results of Bergmann (1958). However, virus-infected roots were noticeably thicker than healthy roots. TMV-infected root cultures began to show necrosis of the 2–3 cm at the cut end by the time they had grown in length to about 150–200 mm.

TMV–RNA was detectable as a uv absorption peak on a gel of nucleic acid extracted from an infected, 200-mm-long root (Fig. 1C). TMV–RNA was calculated from its peak area on the uv absorption scan (Fraser, 1971) to be just over 4% of the total nucleic acid present in the whole 200-mm-long root.

Figures 1A and 1B show typical examples of ^{32}P incorporation into nucleic acids of root segments. DNA synthesis was detectable in all segments of 60-mm-long roots, but only in the apical 100 mm of 200-mm-long roots. The meristem, the region of most active division, is localized in the apical 1 mm of the root. The persistence of DNA synthesis so far back into the root was probably due to endopolyploidy and maintained cell divisions, such as those associated with lateral root formation.

Most of the ^{32}P incorporation into high molecular-weight RNA was into the two ribosomal RNAs. The long chase incubation in ^{31}P-containing medium was necessary to remove radioactivity from the rRNA precursor (Rogers, Loening, and Fraser, 1970) which has an electrophoretic mobility similar to that of TMV–RNA. The gel of healthy root nucleic acids (Fig. 1A) showed no significant labeling of rRNA precursor. The peak of labeling at 30 mm in the gel of nucleic acid from TMV-infected root was, therefore, identified as TMV–RNA. This was substantiated by the identity of its electrophoretic mobility with that of TMV–RNA extracted from purified virus (Fig. 1C).

Its base composition was also found to be that of TMV–RNA.

Incorporation of ^{32}P into RNA at Varying Distances from the Root Tip

The data on the distribution of ^{32}P incorporation shown in Figs. 2 and 3 were measured on single infected and control roots, as this gives a clearer picture of the situation within the individual root. The results presented are typical of several experiments.

The pool of ^{32}P in 60-mm-long healthy roots was high near the tip then fell away with distance from the tip (Fig. 2). In 60-mm-long TMV-infected roots, the pool of ^{32}P in the older part of the root was about five times the level in the control, healthy root. The total ^{32}P uptake by a TMV-infected 60-mm-long root during a 70-min incubation was 15% of the ^{32}P supplied.

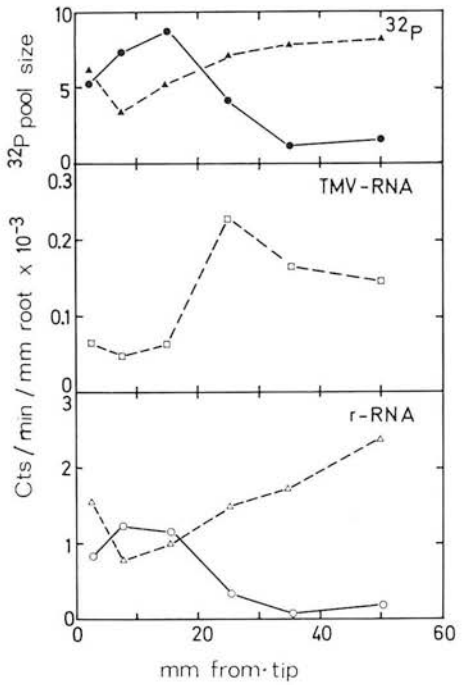

FIG. 2. Changes, with distance from the tip, in 60-mm-long roots, in ^{32}P pool size (relative units) in healthy (●——●) and TMV-infected (▲- -▲) roots; changes in the rate of incorporation of ^{32}P into TMV–RNA in TMV-infected roots (□- -□); and changes in the rate of incorporation of ^{32}P into ribosomal RNA in healthy (○——○) and TMV-infected (△- -△) roots.

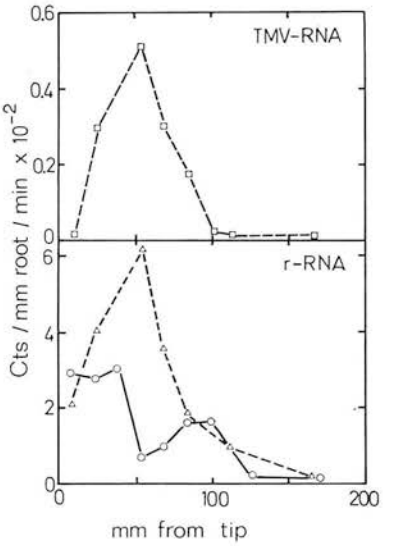

FIG. 3. Changes, with distance from the tip, in 200-mm-long roots, in the rate of incorporation of ^{32}P into TMV–RNA in TMV-infected roots (□- -□), and in the rate of incorporation of ^{32}P into ribosomal RNA in healthy (○——○) and TMV-infected (△- -△) roots.

In 200-mm-long roots, the size of the ^{32}P pool in TMV-infected roots was again higher than in healthy roots for much of the length of the roots. In both healthy and TMV-infected roots, the pool size declined sharply in the region 150–200 mm from the tip.

The incorporation of ^{32}P into TMV–RNA was low in the first 10 or 20 mm of infected 60- and 200-mm-long roots (Figs. 2 and 3). The maximum rate of incorporation of ^{32}P into TMV–RNA was in the region 20–40 mm behind the tip in roots 60 mm long and 20–80 mm behind the tip in 200-mm-long roots. The incorporation of ^{32}P into TMV–RNA declined in the older parts of the root; in the last 100 mm of 200-mm roots, very little ^{32}P labeling of TMV–RNA occurred.

In the healthy, 60-mm-long root, the incorporation of ^{32}P into ribosomal RNA was at a high level in the first few centimeters after the tip, then declined toward the end of the root (Fig. 2). In 200-mm-long roots, there was a similar pattern, but there was a second region of high ribosomal RNA labeling between 60 and 120 mm. This was the region where lateral root initiation was apparent (Fig. 3).

The distribution of incorporation of ^{32}P into ribosomal RNA along TMV-infected roots was in direct contrast to that in healthy roots. The rate of incorporation increased with distance from the tip or from close behind the tip (Figs. 2 and 3), until 50 or 60 mm behind the tip. At this point in the root the rate of incorporation of ^{32}P into ribosomal RNA in infected roots could be as much as 10 times the rate in healthy roots. In 200-mm-long infected roots, the rate of incorporation of ^{32}P into ribosomal RNA declined drastically 60–200 mm behind the tip (Fig. 3).

In 200-mm-long infected roots (Fig. 3), the region of stimulated incorporation of ^{32}P into ribosomal RNA coincided with the location of highest labeling of TMV–RNA. In 60-mm-long infected roots (Fig. 2), incorporation of ^{32}P into ribosomal RNA was high at the point of maximum labeling of TMV–RNA, but continued to increase with distance from the tip, while TMV–RNA labeling declined slightly.

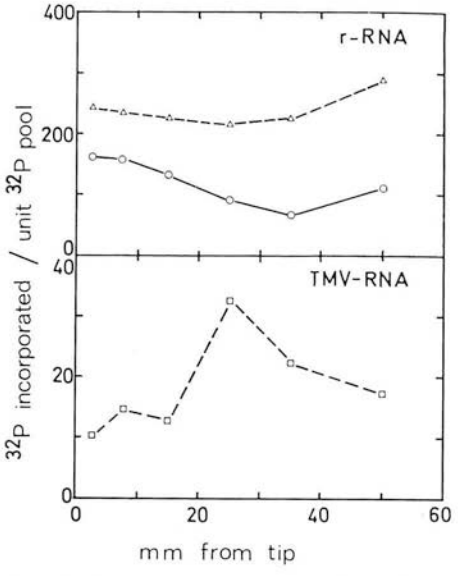

FIG. 4. Changes, with distance from the tip, in 60-mm-long roots, in the incorporation of ^{32}P into RNA, expressed per unit ^{32}P pool size (relative units). ○——○ rRNA in healthy roots; △- -△ rRNA in TMV-infected roots; □- -□ TMV-RNA. These data were derived from the data shown in Fig. 2.

DISCUSSION

We have tried to measure rates of TMV–RNA and ribosomal RNA synthesis in different regions of infected and healthy roots by measuring the incorporation of ^{32}P into RNA. A major problem with this technique is that differences in ^{32}P incorporation could arise in two ways: 1. By changes in the rate of macromolecular synthesis. 2. By changes in the size of precursor pools or the rate of entry of isotope into the pool. For example, it could be argued that the distribution of TMV–RNA labeling along the root (Fig. 2) merely reflects changes in the size of the ^{32}P pool along the root, and that the higher ^{32}P incorporation into ribosomal RNA in virus-infected roots is a result of the ^{32}P pool being larger in infected roots than in control roots. We have two reasons for rejecting these interpretations and regarding the ^{32}P incorporation as a meaningful measure of RNA synthesis.

1. Figure 4 shows the results of Fig. 2 replotted on a per unit pool ^{32}P basis. Clearly the distribution along the root of ^{32}P labeling of TMV–RNA is similar whether the results are expressed as total incorporation (Fig. 2) or as incorporation per unit pool ^{32}P (Fig. 4). Therefore, changes in the rate of TMV–RNA labeling along the root cannot be ascribed to changes in the size of the ^{32}P pool, and are taken to show changes in the rate of TMV–RNA synthesis.

Furthermore, the stimulation (in comparison with control roots) of labeling of ribosomal RNA in the region 20–60 mm from the tip in infected 60-mm roots (Fig. 2) is still observed when the results are replotted on a per unit pool size basis (Fig. 4) despite the fact that the ^{32}P pool in this area of infected roots is larger than in control roots. We take this as an indication that the increased labeling of rRNA in infected roots is a consequence of stimulated rRNA synthesis. It is quite conceivable, and consistent with these results, that the rate of macromolecular synthesis governs the pool size and rate of uptake of ^{32}P, and that the stimulation of RNA synthesis in infected roots is a cause of their increased ^{32}P pool size.

2. It is possible that there is longitudinal

transport of materials in root cultures, and that the sites of uptake and principal incorporation may be separated. It is likely that after a 6-hr chase incubation in nonradioactive phosphate, the distribution of ^{32}P reflects the regions of active incorporation rather than those of rapid uptake.

TMV–RNA Synthesis

The synthesis of TMV–RNA was low in the apical region of the root (Figs. 2 and 3). Previous reports have shown that no virus infectivity can be found in the apical meristems of roots. Smith and Schlegel (1964) found no infectivity whatsoever of clover yellow mosaic virus in the apical 400 μ of *Vicia faba* root tips; Crowley and Hanson (1960) reported very low infectivities in the apical 4 mm of TMV-infected tomato roots. The fineness of analysis used in the present investigation was not sufficient to establish the size of any apical zone free of viral RNA synthesis. What has been shown is that some TMV–RNA synthesis did occur in the first 5 mm behind the apex (Fig. 2). However, the rate of TMV-RNA synthesis did not reach a maximum until relatively far back in the root, at about 40–60 mm behind the tip. This parallels the situation in TMV-infected young tobacco leaves. When very young leaves are infected, the duration of the accumulation of TMV–RNA is much greater than when mature leaves are infected, and the bulk of the TMV–RNA accumulation occurs after considerable leaf expansion has taken place (Fraser, 1972). The results from both root and leaf, therefore, suggest that while TMV is capable of some multiplication in relatively juvenile tissue, the host is more favorable for extensive virus multiplication when rather more mature.

rRNA Synthesis

TMV-infected roots showed levels of ribosomal RNA synthesis considerably in excess of those in healthy roots. The portion of root showing the greatest stimulation of rRNA synthesis was also the region of high TMV–RNA synthesis (Figs. 2 and 3). TMV infection may also stimulate rRNA synthesis in infected, mature tobacco leaves, but this stimulation manifests itself before and after the main period of TMV–RNA accumulation. During the most active TMV–RNA synthesis the rate of rRNA synthesis in infected leaves is depressed, and is considerably lower than in healthy leaves (Fraser, 1973). There is thus a major difference between roots and leaves with respect to rRNA synthesis during TMV–RNA accumulation. It is likely that the explanation lies in the vastly different amounts of TMV–RNA synthesized in the two organs. About 4% of the total nucleic acid of long-term infected root cultures is TMV–RNA (Fig. 1); in leaves, TMV–RNA may be as much as 75% of the total nucleic acid content (Fraser, 1971). ^{32}P incorporation into TMV–RNA in root cultures was one fifth of the incorporation into rRNA in the segment of root showing most TMV–RNA synthesis (Fig. 2) whereas in leaves, the incorporation of ^{32}P into TMV–RNA may be as much as four times the incorporation into rRNA (Fraser, 1973). Clearly the root RNA metabolism is much less turned over to the synthesis of viral RNA than is that of the infected leaf. It is possible that at least part of the inhibition of rRNA synthesis in the infected leaf during active TMV–RNA synthesis is due to competition for nucleotide precursors by TMV–RNA synthesis. The amounts of TMV–RNA synthesis in the root are likely to be too small to exert any significant competitive inhibition of rRNA synthesis.

The means by which TMV infection might stimulate rRNA synthesis in the root, and in mature leaves before and after the major TMV–RNA accumulation, remain to be discovered.

ACKNOWLEDGMENTS

This work was supported by the Max-Planck-Gesellschaft zur Förderung der Wissenschaft. We thank Prof. G. Melchers for facilities and encouragement, and Dr. S. Sarkar for helpful discussions.

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Reprinted from VIROLOGY, Volume 56, No. 1, November 1973

VIROLOGY 56, 379–382 (1973)

TMV-RNA is Not Methylated and Does Not Contain a Polyadenylic Acid Sequence

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Accepted July 25, 1973

Several studies of polyribosomes from TMV (tobacco mosaic virus) infected leaves have suggested that TMV-RNA acts as the messenger RNA in polyribosomes synthesising virus protein (*1–3*). This prompted me to examine TMV-RNA for two other characteristics common to messenger RNAs.

Methylation of RNA occurs by the addition of a methyl group to a base or ribose after synthesis of the polynucleotide chain. The methyl group is donated by methionine, via the active intermediate *S*-adenosyl methionine. Ribosomal RNA and transfer RNA are extensively methylated (*4*) but it is known that messenger RNA is not methylated (*5*). The RNA of the RNA-phage R 17, which acts as messenger for the synthesis of R 17 proteins, is also not methylated (*5*).

Figure 1 shows the radioactive labeling of nucleic acids which occurred when TMV-infected tobacco leaf protoplasts (*13*) were incubated for 18 h with [^{32}P]orthophosphate and the methyl group donor [*Me*-^{3}H]methionine. The very long incubation ensured that no significant proportion of the incorporated radioactivity would be found in the ribosomal RNA precursor which has an electrophoretic mobility similar to that of TMV-RNA (*6*).

The 25 S and 18 S ribosomal RNAs became ^{3}H-labeled. DNA was also ^{3}H-labeled, although the absence of incorporation of ^{32}P into DNA during the incubation shows that no actual DNA synthesis occurred. In contrast, there was no detectable incorporation of ^{3}H radioactivity into TMV-RNA. The large incorporation of ^{32}P into TMV-RNA shows that active TMV-RNA synthesis did occur during the incubation.

The absence of ^{3}H-labeling of TMV-RNA, and also of the 5 S ribosomal RNA (not shown) provides an internal control indicating that the ^{3}H-labeling of 25 S and 18 S ribosomal RNA was by methylation after synthesis. It excludes the possibility that ^{3}H-labeling occurred by the entry of the *Me*-^{3}H into nucleotide precursors of RNA via 1-C intermediary metabolism.

The absence of ^{3}H-labeling of TMV-RNA shows that TMV-RNA, if methylated at all, has fewer methylated sites than could be detected by this technique. We can estimate the minimum detectable number of methylated sites in TMV-RNA by comparing its labeling with that of ribosomal RNA. ^{3}H (methyl) labeling represents the amount of methylation; ^{32}P labeling the amount of synthesis during the radioactive incubation. The ratio ^{3}H:^{32}P labeling thus represents the extent of methylation of the RNA synthesised. In Fig. 1, the ratio for ribosomal RNA was 0.48. For TMV-RNA, the minimum ^{3}H-labeling which would have been recognizable as a peak would be twice the background level of ^{3}H radioactivity on the gel. This level of ^{3}H labeling would give a ^{3}H:^{32}P ratio of 0.1. Plant ribosomal RNA has one methylated site per 70 nucleotides (*17*). Therefore the minimum number of methylated sites which could have been unequivocally detected in TMV-RNA under the conditions of Fig. 1 is one methylation per 335 nucleotides. In an experiment similar to that shown in Fig. 1, but with a radioactive incubation of 48 hr instead of 18 hr, the ^{32}P-labeling of TMV-RNA was correspondingly higher than in Fig. 1, while ^{3}H-labeling was still absent. In this case methylation

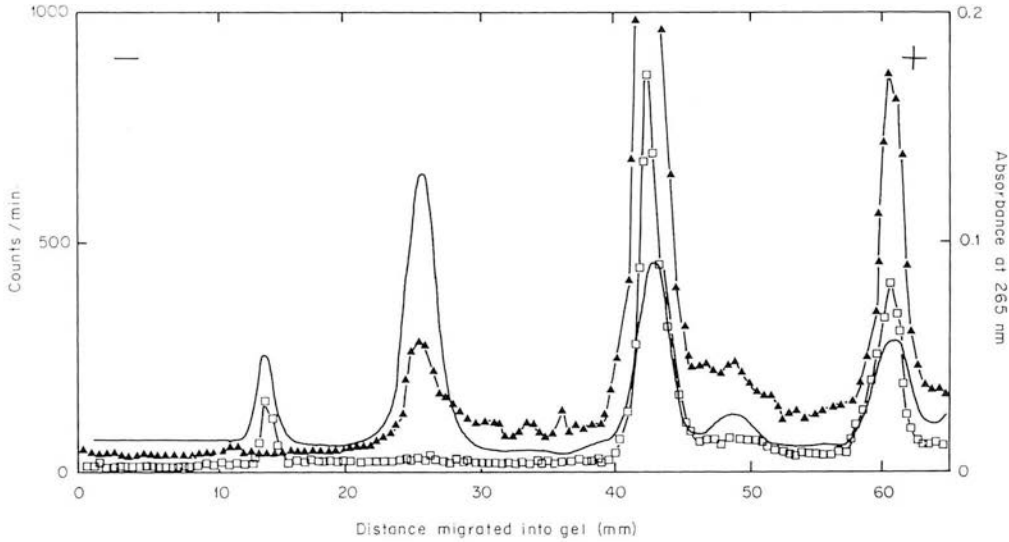

FIG. 1. Polyacrylamide gel electrophoresis of nucleic acids extracted from TMV-infected tobacco leaf cell protoplasts. Protoplasts from 150-mm long leaves of tobacco (*Nicotiana tabacum* L. var. "Xanthi") were prepared, inoculated with TMV strain A14, and cultured by Dr. I. Takebe (*13*). After 10 h of culture at 24 C and 3500 lx Osram "L-Fluora" light, 20 mg protoplasts were washed with phosphate-free culture medium (*13*) and resuspended in 10 ml phosphate-free culture medium containing 500 μCi [*Me*-^{3}H]methionine and 30 μCi [^{32}P]orthophosphate (Radiochemical Centre, Amersham, U. K.). After a further 18 hr incubation the protoplasts were collected by centrifugation and lysed in 30 m*M* Tris–HCl, pH 7.6 at 0°C; 50 m*M* KCl; 5 m*M* $MgCl_2$; 1% sodium tri-isopropylnaphthalene sulfonate, and 6% sodium 4-aminosalicylate. RNA was prepared (*14*) and fractionated by electrophoresis (*15*) on a 2.3% gel for 2 hr at 5 mA and 8 V/cm gel length. The gels were scanned for ultraviolet absorbance (——) in a Joyce-Loebl Chromoscan. The peaks are: at 14 mm, DNA; at 26 mm, TMV-RNA; at 43 mm, 25 S ribosomal RNA; at 60 mm, 18 S ribosomal RNA. The optical density peak for TMV-RNA includes 10 μg of purified TMV-RNA (*14*) added as a marker and fractionated together with the protoplast RNA. The gel was frozen, cut transversely into 0.5 mm slices, and ^{3}H (□——□) and ^{32}P (▲——▲) radioactivity in each slice was determined in a Packard Tri-Carb Liquid Scintillation Counter (*16*). ^{3}H counts were corrected for spill-over of ^{32}P counts into the ^{3}H channel.

could have been detected down to a limit of one methylated site per 1000 nucleotides.

Thus TMV-RNA has fewer than six methylated sites per molecule. This absence or extremely low level of methylation is consistent with a messenger RNA function (*5*).

It has recently become apparent that a characteristic feature of eukaryote messenger RNAs is a sequence rich in adenylic acid residues at the 3′ end. Such polyadenylic acid (poly(A)) sequences have been found in organisms as diverse as mammals (*7–9*), amphibians (Rosbash and Ford, in preparation), yeast (*18*), and tobacco plants (this report). The only eukaryote messengers presently known not to contain poly-A sequences are those for the histones (*10*).

RNA molecules containing poly(A) sequences can be detected and characterized by hybridizing tritiated complementary homopolynucleotide, poly[^{3}H] uridylic acid (poly([^{3}H]U)) to the poly(A) regions in total RNA, then fractionating the mixture by sedimentation on a sucrose gradient. The distribution of the radioactivity on the gradient then shows the distribution of those molecules containing poly(A) sequences. This technique is described in detail elsewhere (*11*). Figure 2A shows its application to tobacco leaf RNA.

When poly([^{3}H]U) was sedimented alone, it remained near the top of the gradient. Its sedimentation coefficient was about 3 S. When mixed with tobacco leaf RNA, most of the poly(U) radioactivity was carried down

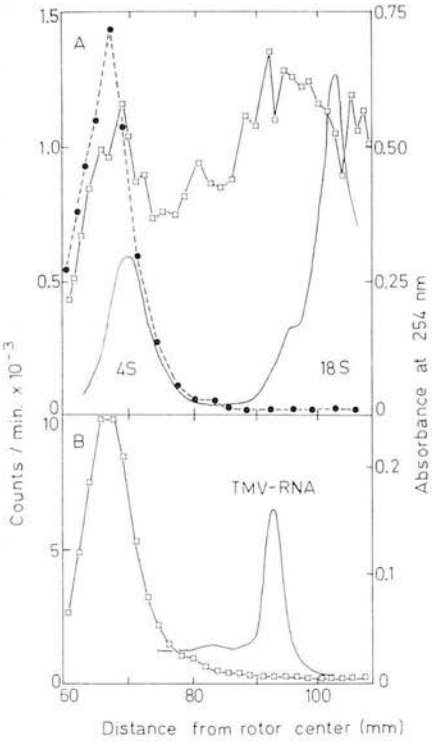

FIG. 2. Sucrose density gradient sedimentation of mixtures of tobacco leaf RNA or TMV-RNA with poly[^{3}H]uridylic acid. The gradients were linear 7–25% sucrose in 50 m*M* Tris–HCl pH 7.6; 150 m*M* NaCl; 1 m*M* EDTA, and 0.5% sodium lauryl sulfate. One tenth milliliter of sample in this buffer minus sucrose was applied to a 5-ml gradient which was centrifuged in the SW 50.1 rotor of the Spinco L50 centrifuge for (A) 4 hr at 50,000 rpm or (B) 1.5 hr at 45,000 rpm, at 18 C. After centrifugation, ultraviolet absorbance at 254 nm was determined by pumping the gradient through an ISCO monitor. 6-Drop fractions were collected and the radioactivity in each determined (*11*). (A) 55 μg of total RNA extracted (*14*) from a 15-cm long tobacco leaf was mixed with 0.25 μg poly[^{3}H]uridylic acid (Miles). The continuous line shows ultraviolet absorbance; (□——□) radioactivity. (●--●) shows the distribution of radioactivity when poly([^{3}H]U) was centrifuged alone. (B) 25 μg of TMV-RNA extracted (*14*) from purified virus was mixed with 0.2 μg poly([^{3}H]U). (——) ultraviolet absorption; (□——□) radioactivity.

to heavier regions of the gradient. This shows that the leaf contained a heterogeneous population of RNAs which contained poly(A) regions, ranging in size from 4 S to more than 20 S. When TMV-RNA, prepared from purified virus, was mixed with poly([^{3}H]U) and sedimented, the poly([^{3}H]U) remained at the top of the gradient; none associated with the TMV-RNA (Fig. 2B). When TMV-RNA was heated to 60 or 80 C, to melt any internal double-stranded regions, then cooled with poly([^{3}H]U) present, the results obtained on fractionation of the mixture were identical to those shown in Fig. 2B. This excludes the possibility that a poly(A) sequence in TMV-RNA might be masked by pairing with a poly(U) sequence also in the TMV-RNA molecule.

Therefore TMV-RNA does not contain a poly(A) sequence. This is in direct contrast to most known messengers, including the heterogeneous RNA (presumed to be messenger) of tobacco leaf. The mRNA of vaccinia virus has been shown to contain a poly(A) region (*12*). However, the absence of poly(A) sequences from histone messenger RNA (*10*) shows that poly(A) is not absolutely required for messenger activity. Therefore the absence of a poly(A) sequence in TMV-RNA does not mean that TMV-RNA cannot be a messenger.

Experiments are in progress to test the possibility that those TMV-RNA molecules associated with polyribosomes are different from those incorporated into virus particles. The experiments reported here do not exclude the possibility that poly(A) sequences are present in those TMV-RNAs associated with polyribosomes.

ACKNOWLEDGMENTS

This work was supported by the Max-Planck-Gesellschaft zur Förderung der Wissenschaft and by the Medical Research Council. I thank Prof. G. Melchers for facilities; Dr. S. Sarkar for TMV; Dr. I. Takebe for infecting tobacco leaf protoplasts, and Dr. C. Leaver for tobacco plants.

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J. gen. Virol. (1978), **39**, 191–194
Printed in Great Britain

Inhibition of the Multiplication of Tobacco Mosaic Virus by Methyl Benzimidazol-2-yl Carbamate

(*Accepted* 11 *November* 1977)

SUMMARY

Methyl benzimidazol-2-yl carbamate (MBC, carbendazim) is the fungitoxic principle of the systemic fungicide benomyl. It reduces the severity of symptoms produced by tobacco mosaic virus infection of tobacco plants (Tomlinson *et al.* 1976). Measurements of the accumulation of TMV RNA in MBC-treated and control plants showed that MBC treatment significantly reduced the accumulation of virus, by 20 % in lower leaves and by up to 90 % in upper leaves. No phytotoxic effects of MBC were observed at the concentrations effective against virus multiplication. Possible modes of action of MBC are discussed.

Methyl benzimidazol-2-yl carbamate (MBC, carbendazim) is the water decomposition product and fungitoxic element of the systemic fungicide benomyl. Recently, Tomlinson *et al.* (1976) showed that MBC reduces the severity of disease symptoms caused by tobacco mosaic virus (TMV) in tobacco plants. In this paper we report that MBC inhibits the multiplication of TMV in systemically-infected tobacco plants.

Nicotiana tabacum L. cv. White Burley plants were grown in John Innes No. 2 compost in 12·5 cm diam. pots. The plants were grown under natural lighting conditions in a glass house at 20 to 25 °C. Plants with three fully expanded leaves were watered with 200 ml of a 5 g/l suspension of 'Bavistin' (BASF 3460F), a commercial formulation containing 50 %, w/w, MBC and 50 % non-active filler. Seven days later, a second application of Bavistin was made: each plant thus received a total of 1 g of MBC. There was no sign of any toxic effects of MBC treatment. Plants of the same size treated with up to ten times the dose also showed no ill effects or inhibition of growth.

Two days after the second application of MBC, the two highest fully-expanded leaves on each plant were dusted with 400 mesh Carborundum and inoculated by rubbing with a 0·1 %, w/v, solution of TMV strain 'Grapevine' in 50 mM-sodium phosphate buffer, pH 7·0. Control plants which had not received MBC were also inoculated.

TMV multiplication was measured by the accumulation of TMV RNA. Samples of 0·5 to 1·0 g of infected leaf were homogenized in 10 ml extraction medium [2 %, w/v, sodium tri-isopropyl naphthalene sulphonate; 50 mM-tris-HCl, pH 7·8; 10 mM-NaCl; 0·2 %, v/v, 2-mercaptoethanol; 0·2 %, v/v, silicone DC antifoam emulsion (Dow Corning)] in an Ultra Turrax homogenizer. Protein was removed from the homogenate by treatment with phenol-chloroform-*m*-cresol (Fraser, 1975). Total nucleic acid was precipitated with ethanol and further purified by dialysis against 0·5 %, w/v, sodium dodecyl sulphate; 150 mM-sodium acetate at pH 6·0 (Fraser, 1969). Samples of 10 to 15 μg total nucleic acid were fractionated by electrophoresis on 2·2 % polyacrylamide gels (Loening, 1969) for 3 h at 8 V/cm. The gels were scanned for absorbance at 265 nm in a Joyce Loebl Gel Scanner. TMV RNA was detected as a prominent peak running between the DNA and the larger ribosomal RNA. No such peak was detected on gels of nucleic acids from healthy leaves.

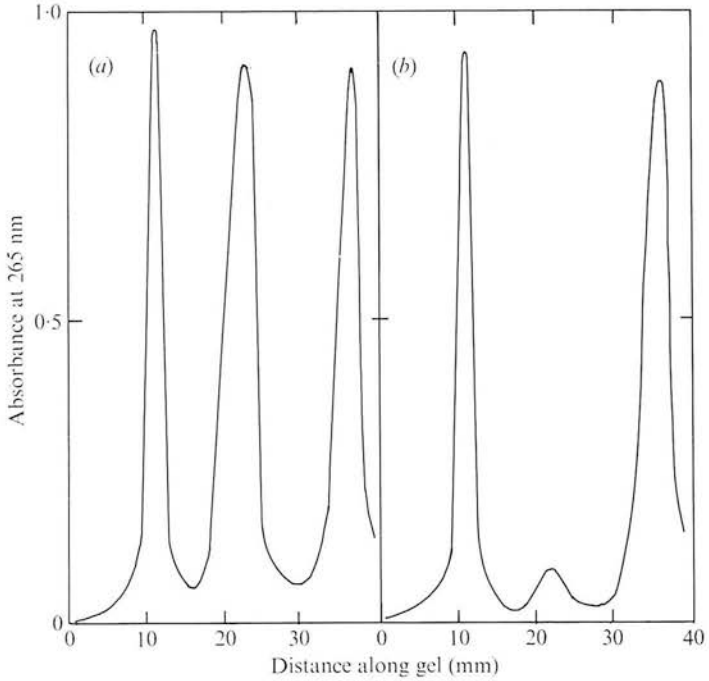

Fig. 1. Polyacrylamide gel electrophoresis of nucleic acids from tobacco leaves infected with TMV. The ultraviolet absorption peaks are DNA at 11 mm; TMV RNA at 20 to 22 mm and the larger (25S) ribosomal RNA at 37 mm. The nucleic acids were extracted from the systemically-infected ninth leaves above the inoculated leaves, 28 days after inoculation. The scans represent electrophoresis of nucleic acids from equal weights of leaf material: (*a*) control, (*b*) MBC-treated.

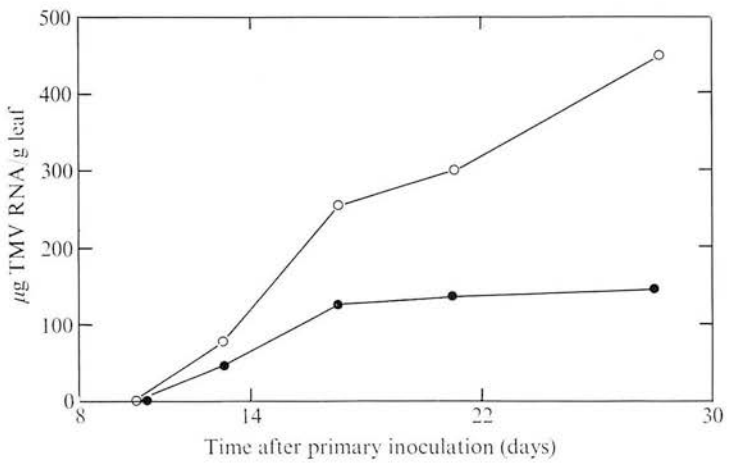

Fig. 2. Changes in TMV RNA content per g fresh weight with time in the systemically-infected fifth leaf above the inoculated leaves. ○—○, Control; ●—●, MBC-treated. Day o is the time of inoculation of the lower leaves.

The identity of the TMV RNA peak was confirmed by co-electrophoresis with authentic TMV RNA prepared from purified virus. The TMV RNA content of the leaf was calculated from the area of the TMV RNA ultraviolet absorption peak: peak area is linearly proportional to weight of RNA in the peak (Fraser, 1971).

The nucleic acid extraction and purification method used gave yields of over 90 % of the total leaf nucleic acid content, measured chemically as explained elsewhere (Fraser, 1971). Determinations of TMV RNA content on replicate samples taken from the same batch of infected leaf material indicated that reproducibility was high: all individual determinations were within ± 10 % of the mean.

Fig. 1 shows the nucleic acids extracted from comparable systemically-infected upper leaves of control and MBC-treated plants, 28 days after primary infection. The amount of TMV RNA in the MBC-treated leaf was only about 10 % of that in the control: MBC therefore strongly inhibited virus multiplication. In contrast, there was no significant effect of MBC on the accumulation of the host nucleic acids: the peak areas of the DNA and 25S ribosomal RNA were similar in gels of nucleic acids from control and MBC-treated leaves (Fig. 1). In these upper leaves, most of the growth and nucleic acid accumulation occurred during the time the plants were exposed to MBC. E. M. Faithfull (personal communication) has shown that MBC persists in White Burley tobacco leaves for 45 days after application.

The extent of inhibition of TMV multiplication by MBC treatment depended on the height of the leaf on the plant. The 90 % inhibition of TMV RNA synthesis shown in Fig. 1 occurred in the ninth leaf above the upper inoculated leaf. In the fifth leaf above the inoculated leaf, MBC treatment inhibited TMV multiplication by about 60 % (Fig. 2). In the inoculated leaves themselves, TMV multiplication was inhibited only 20 to 30 % by MBC treatment. The different levels of inhibition of TMV RNA accumulation in upper and lower leaves were probably not caused by differences in MBC concentration in different parts of the plant. J. A. Tomlinson (personal communication) has shown that the highest concentrations of MBC in the plant occur in the lower leaves.

The inhibition of TMV RNA accumulation in the upper leaves by MBC treatment was long lasting. Fig. 2 shows that the rate of TMV RNA accumulation in treated leaves decreased earlier than in control leaves. The TMV RNA content remained fairly constant for a period of at least 10 days following the phase of virus RNA accumulation in MBC-treated leaves. Tomlinson *et al.* (1976) showed that 30 days after inoculation, the infectivity of the non-dark green areas of infected control leaves was higher than that of leaves from MBC-treated plants. One hundred days after inoculation, however, they found similar levels of infectivity in the uppermost systemically infected leaves of MBC-treated and control plants.

The means by which MBC treatment restricts TMV multiplication are not known. Fig. 2 shows that TMV RNA synthesis was measureable in systemically-infected leaves at the same time after inoculation of lower leaves in control and MBC-treated plants. It is thus unlikely that MBC is slowing the systemic spread of virus through the host. Other benzimidazole derivatives have been shown to inhibit animal virus RNA polymerase (Tamm & Eggers, 1963) and initiation of heterogeneous nuclear RNA synthesis by RNA polymerase II in HeLa cells (Sehgal *et al.* 1976): it is conceivable that MBC inhibits TMV RNA synthesis directly. MBC also exhibits cytokinin-like activity (Thomas, 1974): it is possible that inhibition of virus RNA synthesis is mediated through growth regulator metabolism. Effects of cytokinins on infectivity and local lesion size have been reported (Milo & Srivastava, 1969; Aldwinckle, 1975).

We thank John Tomlinson for useful discussions and Su Loughlin for capable technical assistance.

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(*Received* 28 *September* 1977)

Reprinted from *Physiological Plant Pathology* (1978) **13**, 51–64

Chemotherapy of plant virus disease with methyl benzimidazol-2yl-carbamate: effects on plant growth and multiplication of tobacco mosaic virus

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Physiological Plant Pathology (1978) **13**, 51–64

Chemotherapy of plant virus disease with methyl benzimidazol-2yl-carbamate: effects on plant growth and multiplication of tobacco mosaic virus

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(*Accepted for publication January 1978*)

Methyl benzimidazol-2yl-carbamate inhibited the accumulation of tobacco mosaic virus in actively growing parts of infected tobacco plants. It also prevented the inhibition of leaf growth by the virus. In expanding leaves, phytotoxic effects of methyl benzimidazol-2yl-carbamate were seen only at levels 50 to 100 times those required to inhibit viral multiplication. No direct inhibition of tobacco mosaic virus ribonucleic acid synthesis was found when the effects of methyl benzimidazol-2yl-carbamate on incorporation of ^{32}P-orthophosphate into nucleic acid were studied. From experiments on time of application, dose level and leaf age, it is suggested that methyl benzimidazol-2yl-carbamate inhibits viral ribonucleic acid synthesis indirectly, by maintaining the host in a state unsuitable for viral multiplication. This may occur through the known cytokinin activity of the compound. In some circumstances, higher concentrations of tobacco mosiac virus were found to result from treatment with methyl benzimidazol-2yl-carbamate. The relationships between leaf growth, symptom formation and viral multiplication are discussed.

INTRODUCTION

There have been several reports of chemicals which suppress the multiplication of plant viruses [*5*, *7*, *15*, *18*, *20*, *26*, *28*]. These compounds are generally unsuitable for use on crops because of their cost and toxicity to animals. Many are also toxic to the host plant at concentrations little above those required to inhibit viral multiplication. Recently, Tomlinson *et al.* [*25*] showed that the visible symptoms of tobacco mosaic virus (TMV) infection of tobacco plants could be suppressed by treating the plants with non-phytotoxic doses of methyl benzimidazol-2yl-carbamate (MBC, carbendazim). This compound is the water decomposition product and fungitoxic principle [*6*] of the systemic fungicide benomyl [methyl 1-(butylcarbamoyl)-benzimidazol-2yl-carbamate], which is already in wide use in agriculture.

Fraser & Whenham [*14*] showed that the multiplication of TMV in young leaves of infected tobacco plants was much reduced by MBC treatment. Bailiss *et al.* [*2*] found both increased and decreased infectivity in TMV-infected tomato leaf discs floated on benomyl solutions of different concentrations.

This paper describes further experiments on the effects of MBC on TMV-infected

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tobacco plants. The relationships between viral multiplication, symptom development and leaf growth are considered, and a possible mechanism for the effects of MBC on viral multiplication is suggested.

MATERIALS AND METHODS

Host plants and viruses

Tobacco plants (*Nicotiana tabacum* L.) cvs White Burley and Samsun were grown in John Innes no. 2 compost in 12·5 cm diameter pots. The plants were kept in a glasshouse under natural summer lighting conditions. The temperature was 17 °C night and 20 to 25 °C day.

Methyl benzimidazol-2yl-carbamate (MBC) was supplied as "Bavistin" (B.A.S.F. U.K. Ltd, Hadleigh, Suffolk), a commercial formulation containing 50% w/w MBC in non-active filler [*25*]. Plants were watered with 200 ml suspension containing various concentrations of MBC. Times of application of MBC are given in Results.

Tobacco mosaic virus (TMV) strains Rothamsted, Vulgare and Grapevine were used. The first two give a mild light green/dark green mosaic; Grapevine causes more severe yellowing symptoms. Inocula were prepared by grinding frozen, infected White Burley leaf tissue with a pestle and mortar. Leaves were dusted with 400 mesh carborundum and inoculated by rubbing with a 1000-fold dilution of inoculum in 50 mM phosphate buffer, pH 7·0. Viral multiplication was followed in the inoculated leaves and also in the younger, upper leaves, which became systemically infected.

Measurement of TMV multiplication

TMV multiplication was measured by the accumulation of TMV RNA in infected leaves. Samples of 0·5 to 1·0 g tissue were homogenized for 5 min in 10 ml extraction medium [2% w/v sodium tri-isopropylnaphthalene sulphonate; 50 mM Tris–HCl pH 7·8; 10 mM NaCl; 0·2% v/v 2-mercaptoethanol; 0·2% v/v silicone DC antifoam emulsion (Dow Corning)] in an Ultra-Turrax homogenizer operated at one-quarter speed. The homogenate was deproteinized by treatments with phenol-chloroform-*m*-cresol [*11*]. Total nucleic acid was precipitated by ethanol and further purified by reprecipitations and dialysis [*9*].

Samples of 10 to 15 μg total nucleic acid were fractionated by electrophoresis for 3 h at 8 V cm^{-1} on polyacrylamide gels of final acrylamide concentration 2·1% [*17*]. The gels were scanned for absorbance at 265 nm using a Joyce Loebl Gel Scanner. TMV RNA content of the sample, and hence per g leaf, was calculated from the area of the TMV RNA ultraviolet absorption peak on the scan. Peak area is linearly proportional to weight of RNA [*9*]. Details of the yield and reproducibility of the extraction and assay method are given elsewhere [*9*, *14*]. The leaf content of the larger ribosomal RNA (25 S rRNA) was measured in the same way. Each assay of TMV RNA or 25 S rRNA was performed at least in triplicate.

Infectivity of leaf material was measured by local lesion assay on half-leaves of *N. glutinosa* L. Infected leaf samples were ground in a pestle and mortar and diluted to 1 in 10^4 with 50 mM sodium phosphate buffer, pH 7·0, before inoculation onto ten half-leaves. Lesions were counted 5 days after inoculation.

Rates of TMV RNA and 25 S rRNA synthesis

Leaf discs of 10 mm diameter were punched from the lamina of infected leaf material using a cork borer. Six discs were floated on 5 ml water containing various concentrations of technical grade methyl benzimidazol-2yl-carbamate (Stauffer Chemicals, Geneva, Switzerland). The discs were infiltrated under vacuum for 2 min then incubated in a controlled environment cabinet at 25 °C and 15 klx fluorescent light. After 1 h, 0·5 mCi ^{32}P-orthophosphate (Radiochemical Centre, Amersham, U.K., specific activity 100 Ci/mg) was added to each treatment and the incubation continued for 4 h. Nucleic acids were extracted from the discs as above. Total ^{32}P uptake by the discs was measured [*13*]. The nucleic acids were fractionated by polyacrylamide gel electrophoresis and ^{32}P incorporation into TMV RNA and 25 S rRNA determined [*12*].

Chlorophyll content

Samples of 0·5 to 1·0 g of leaf were extracted with 80% v/v acetone and the content of total chlorophyll, chlorophyll *a* and chlorophyll *b* measured spectrophotometrically [*27*].

RESULTS AND DISCUSSION

Effects of MBC on TMV multiplication

Figure 1 shows the time course of TMV RNA accumulation in leaves of control and MBC-treated plants. In the lower leaves, inoculated when they were almost fully expanded, MBC treatment had little or no effect on the pattern or amount of TMV RNA accumulation. In the higher, systemically infected leaves, however, MBC treatment reduced the level of TMV RNA accumulated.

This difference in effect of MBC was not a difference in effect on inoculated and systemically infected leaves as such—Fig. 2 shows that the inhibition of TMV RNA accumulation by MBC increased progressively with height of the leaf on the plant. In the tenth leaf above the inoculated leaf, TMV RNA concentration in leaves of MBC-treated plants was less than 2% of that in control plants, 30 days after inoculation. The pattern of increasing MBC inhibition of TMV RNA accumulation with height of leaf on the plant was found in plants inoculated with the Rothamsted strain of TMV, or with the Grapevine strain which produces more severe visible symptoms.

In leaves in which the accumulation of TMV RNA was reduced by MBC treatment, there was also a reduced level of infectivity (Table 1). Similarly, Tomlinson *et al.* [*25*] reported a lower level of infectivity in leaves of MBC-treated plants than in non-dark green areas of infected control plant leaves. Bailiss *et al.* [*2*] found a slightly lower level of infectivity in TMV-infected tomato leaf discs floated on low concentration solutions of benomyl.

These results raise two questions: how does MBC treatment inhibit TMV RNA accumulation in the upper leaves, and why does it fail to inhibit TMV RNA accumulation in the lower leaves? We first tested the hypothesis that there might be uneven uptake of MBC into different parts of the plant, and hence uneven effects on viral multiplication. Plants were exposed to a wide range of doses of MBC in an attempt

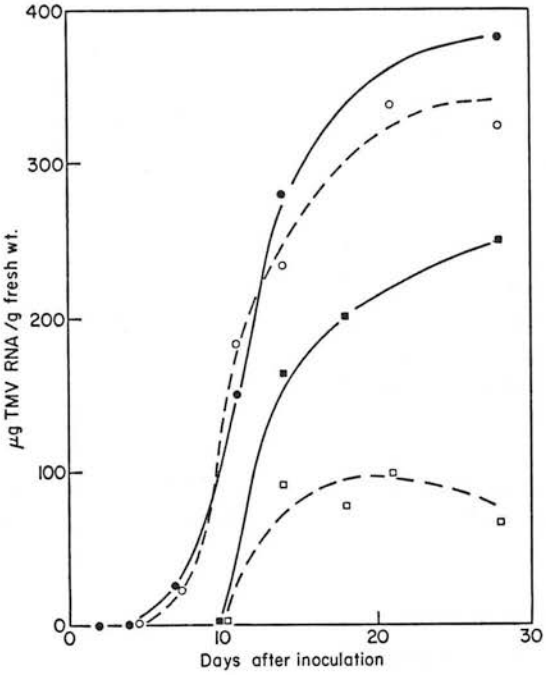

FIG. 1. Effects of MBC on accumulation of TMV RNA in leaves of White Burley tobacco. Plants were treated with 0·5 g MBC 9 and 2 days before inoculation of the uppermost fully expanded leaf with the Rothamsted strain of TMV. TMV RNA was measured in inoculated leaves of MBC-treated (○ - - ○) and control (●——●) plants, and in the fifth leaf above the inoculated leaf in MBC-treated (□ - - □) and control (■—■) plants.

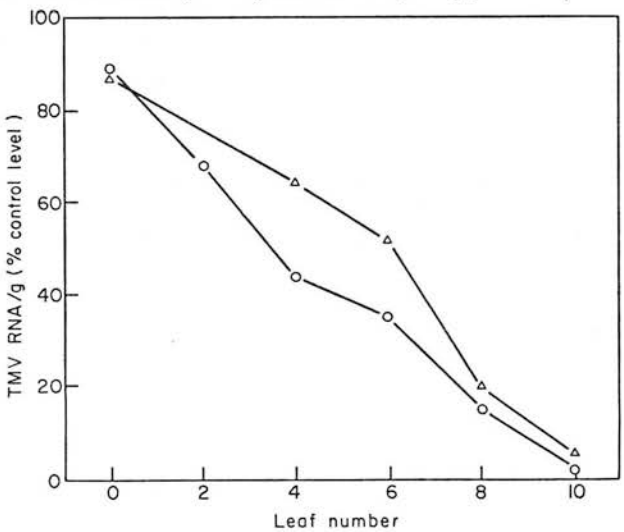

FIG. 2. Effects of MBC on accumulation of TMV RNA in White Burley tobacco plants. Plants were treated with 0·5 g MBC 9 and 2 days before inoculation of the uppermost fully expanded leaf (leaf 0) with TMV strains Rothamsted or Grapevine. 30 days after inoculation, the concentration of TMV RNA in leaves from the inoculated leaf upwards was measured. Values are expressed as a percentage of the TMV RNA concentrations in comparable leaves of plants not treated with MBC. (○—○) Rothamsted; (△—△) Grapevine.

TABLE 1
Infectivity and TMV RNA contents of leaves from control and MBC-treated plants

	Treatment[a]	
	MBC	Control
Infectivity: lesions per half-leaf of *Nicotiana glutinosa*[b]	245 ± 32	366 ± 31
μg TMV RNA/g leaf[b]	193 ± 9	277 ± 10

[a] MBC-treated plants received 0·5 g MBC 9 and 2 days before inoculation of two fully expanded lower leaves with TMV strain Vulgare. White Burley tobacco plants were used.

[b] Infectivity and TMV RNA content of the systemically infected fourth leaf above the upper inoculated leaf were measured 21 days after inoculation. The differences in infectivity and TMV RNA content between MBC-treated and control leaves are significant at the $P = 0{\cdot}02$ level. A log-transformation of the lesion number data was performed [*16*] before testing for significance.

to alter the internal MBC levels, and effects on leaf growth and TMV RNA accumulation in lower and upper leaves were followed.

MBC dose–response curves

Young White Burley tobacco plants with two leaves expanded to about 10 cm long were treated with various levels of MBC. Nine days later the plants received a second treatment at the same level. The total dose applied per plant ranged from 0·1 to 20 g. Fourteen days after the first application of MBC, the two highest fully expanded leaves on each plant (20 cm long) were inoculated with TMV strain vulgare. Twenty-eight days after inoculation, the fresh wts and TMV RNA contents of the inoculated leaves, and of the systemically infected fifth and sixth leaves above were measured.

Figure 3 shows that the growth of the lower, inoculated leaves was severely inhibited when plants were treated with 5 g or more MBC. In plants treated with 10 g MBC, no leaf growth occurred after the first application. Leaves on plants treated with 20 g MBC actually lost weight during the period of treatment, due to severe wilting. It should be noted that the amount of MBC which will inhibit leaf growth is not constant, but depends on the size of plant treated. Plants with three fully expanded leaves at the time of the first application of MBC show inhibition of growth only when treated with doses of 20 g or more [*14*].

The effects of MBC on accumulation of TMV RNA in the inoculated leaves were complex, but replicate experiments showed the same form of dose–response profile. Two examples are shown in Fig. 3. In plants treated with 1 or 2 g MBC, TMV RNA concentration equalled or was slightly lower than in untreated control plant leaves (cf. Figs 1 and 2). With higher levels of MBC treatment, the concentration of TMV RNA reached was higher than in control plant leaves. This elevated concentration of virus was due to the severe inhibition of leaf fresh wt increase by high levels of MBC. Adjusting the TMV RNA per g fresh wt data to a constant total leaf weight basis allows some estimation of the effects of high levels of MBC on viral multiplication unobscured by side-effects of MBC on leaf growth. The adjusted data presented in Fig. 3 indicate that there is progressively increasing inhibition of

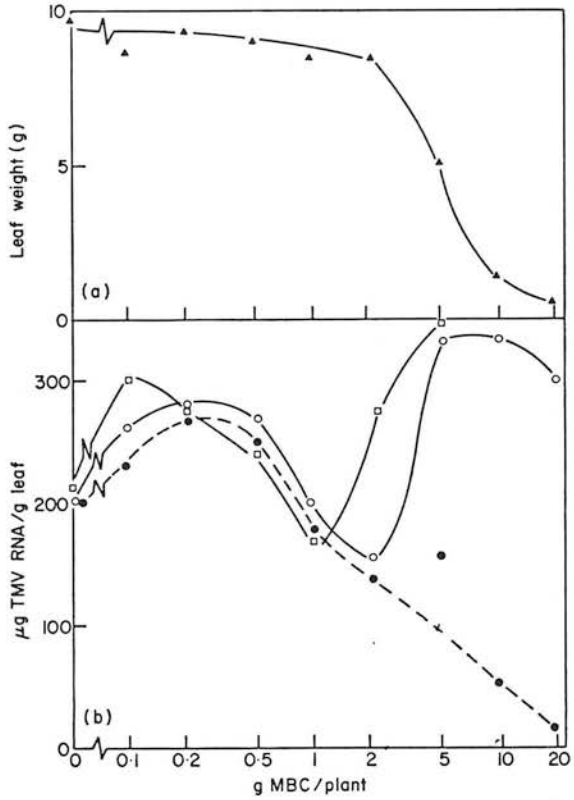

FIG. 3. Effects of dose level of MBC on growth and TMV multiplication in lower leaves of White Burley tobacco. Plants were given various doses of MBC 14 and 5 days before inoculation of the two highest fully expanded leaves with TMV strain vulgare. Fresh wt and TMV RNA content of the inoculated leaves were measured 28 days after inoculation. (a) Fresh wt (▲—▲). (b) TMV RNA concentrations from two separate experiments (○—○) and (□—□). TMV RNA concentration data shown (○—○) were also adjusted to a constant leaf fresh wt basis, to allow some estimation of the effects of MBC on TMV RNA accumulation in isolation from the effects of MBC on leaf fresh wt. The adjustment was performed by multiplying the observed TMV RNA concentration by the observed leaf weight, then dividing by the constant leaf weight. The adjusted data are shown (● - - ●).

TMV RNA accumulation at higher levels of MBC treatment. Thus TMV RNA synthesis can be inhibited by MBC treatment in lower leaves, but only by concentrations of MBC sufficiently high to cause even more severe inhibition of leaf growth.

In plants treated with low doses of MBC, in the range 0·1 to 0·5 g, the TMV RNA concentration in inoculated lower leaves was higher than in comparable leaves of untreated control plants (Fig. 3). This increased concentration could result from stimulation by some means of TMV RNA synthesis in the inoculated leaves by low levels of MBC. Alternatively, the increased concentration of TMV RNA in inoculated leaves might occur as a consequence of an effect of low levels of MBC on the systemically infected upper leaves. It has been shown that one factor limiting the production of TMV RNA in lower, older leaves is the competition for precursors by

synthetic activity in the upper leaves [*10*]. It will be shown below that MBC treatments of 0·1 to 0·5 g per plant strongly reduce the level of TMV RNA synthesis in upper leaves. This reduction in competition may allow the increased TMV RNA accumulation in lower leaves.

In contrast to the results shown in Fig. 3, Bailiss *et al.* [*2*] found that low levels of benomyl slightly reduced the level of infectivity reached in excised, TMV-infected tomato leaf discs, while high concentrations of benomyl caused increased infectivity. The different experimental systems used, and the different methods used to assay TMV, may have contributed to this apparent contradiction.

Figure 4 shows the effects of various doses of MBC on growth and TMV RNA concentration in upper, systemically infected leaves. The average weight of comparable leaves on uninfected control plants was 2 g. TMV infection of plants which received no MBC therefore reduced growth of the upper leaves by almost one half. In plants treated with between 0·1 and 5 g MBC, leaf fresh wt was greater than in the

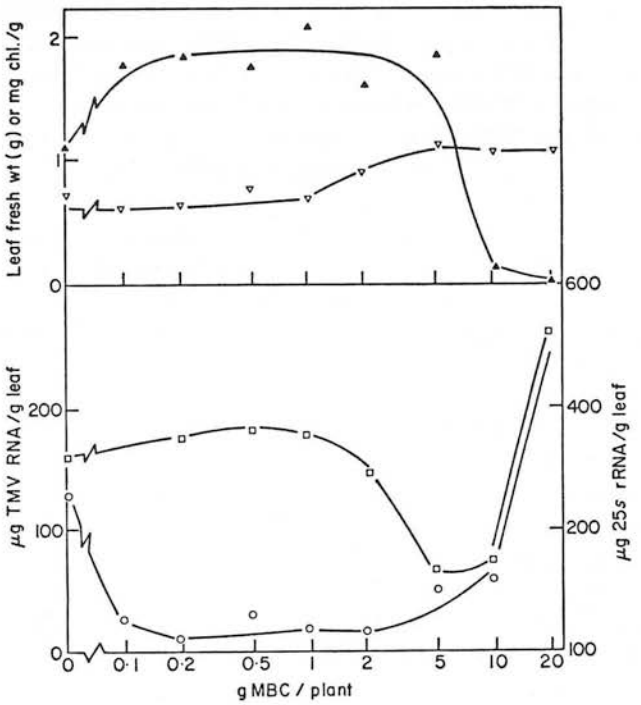

FIG. 4. Effects of dose level of MBC on growth, TMV multiplication, 25 S ribosomal RNA content and chlorophyll content of upper leaves of White Burley tobacco. Plants were treated with MBC and inoculated as in Fig. 3. Twenty-eight days after inoculation, the fifth and sixth systemically infected leaves above the inoculated leaves were harvested. (▲—▲) Mean leaf fresh wt; (▽—▽) total chlorophyll/g leaf; (○—○) TMV RNA/g leaf; (□—□) 25 S rRNA/g leaf.

inoculated, untreated control, and almost equal to that of the healthy control. Thus MBC treatment at these levels largely eliminated the inhibition of leaf growth resulting from viral infection [*10*]. Leaf growth in plants treated with 10 or 20 g MBC was severely inhibited.

In plants treated with between 0·1 and 10 g MBC, the concentration of TMV RNA in the upper leaves was much lower than in untreated control plants (Fig. 4). In plants treated with 20 g MBC, TMV RNA concentration in the upper leaves was high. As in lower leaves, this was a consequence of severer inhibition of leaf growth masking the inhibition of viral multiplication.

In order to test whether the inhibition of TMV RNA accumulation in plants treated with between 0·1 and 10 g MBC was a specific effect, the concentration of the host 25 S rRNA was also measured. MBC doses of 0·1 to 2 g per plant did not alter the 25 S rRNA concentration significantly from the control level. The concentration was lower in plants treated with 5 or 10 g MBC, probably a consequence of the onset of general inhibition of leaf growth at high levels of MBC. This comparison of the effects of MBC on TMV RNA and host RNA contents suggests that the inhibition of TMV RNA accumulation was by some relatively specific mechanism, and not merely by a general inhibition of nucleic acid synthesis.

A comparison of the results for lower (Fig. 3) and upper infected leaves (Fig. 4) shows that growth of both was inhibited by similar doses of MBC. In contrast, accumulation of TMV RNA was inhibited by low levels of MBC in upper leaves, but only by very high levels of MBC in lower leaves. These results suggest that the effects of MBC on growth and TMV RNA accumulation in lower and upper leaves cannot be explained simply as a difference in amounts of MBC taken up into different parts of the plant. This conclusion is supported by direct measurements of MBC contents of lower and upper leaves, which show that the concentration of MBC in lower leaves is actually higher than in upper leaves (Tomlinson, Faithfull & Ward, unpublished results). We therefore require some explanation other than concentration to account for the failure of MBC to inhibit TMV RNA accumulation as effectively in lower leaves as in upper leaves.

Effect of time of application of MBC

One difference between lower and upper leaves in the experiments described above is that lower leaves were first exposed to MBC at a much later stage in their development than younger, upper leaves. We therefore examined whether the stage of leaf development at the time of first application of MBC could influence the effects of MBC on TMV RNA accumulation. Plants were treated with MBC at various times before and during TMV multiplication in lower leaves. Table 2 shows the times and doses of MBC applied, and the TMV RNA concentrations reached in the inoculated lower leaves.

In lower leaves from plants treated with 1 g MBC at 9 days and again at 2 days before inoculation (Treatment A), TMV RNA concentration was slightly higher than in leaves of untreated control plants (Treatment B). The difference was not statistically significant. However, in plants treated with the same total dose of MBC as Treatment A (2 g), but starting 30 days before inoculation (Treatment C), the concentration of viral RNA in the lower leaves was less than half of that in Treatments A or B. When MBC was applied shortly before inoculation and again during the period of TMV multiplication (Treatment D), the TMV RNA concentration reached in the inoculated leaves was the same as when MBC was only supplied shortly before inoculation (Treatments A and E). These experiments therefore show

TABLE 2

Leaf fresh wt and accumulation of TMV RNA in plants treated with MBC at various times before inoculation and during TMV multiplication

Treatment:	A	B (control)	C	D	E
	MBC supplied per plant (g)				
Day of treatment					
−30[a]			0·5		
−20			0·5		
−9	1		0·5	0·5	0·5
−2	1		0·5	0·5	0·5
+10				0·5	
+20				0·5	
Leaf fresh weight (g)[b]	9·17 ± 1·64	10·72 ± 0·60	7·61 ± 1·69	9·42 ± 1·15	9·61 ± 1·91
μg TMV RNA/g leaf[b]	293 ± 30	251 ± 19	112 ± 8	233 ± 13	254 ± 16

[a] At day −30, plants had leaves up to 6 cm long. At day 0, the highest fully expanded leaf on each plant (20 cm long) was inoculated with TMV strain vulgare. Plants were cv. White Burley.

[b] Fresh wt and leaf content of TMV RNA were measured 22 days after inoculation. All values are means ± standard errors. The TMV RNA/g leaf data were adjusted to a constant leaf fresh wt basis, by multiplying the measured concentration by the leaf weight, then dividing by the constant weight (10 g). The TMV RNA concentration in Treatment C is significantly different from all other treatments at the $P = 0{\cdot}01$ level. All other treatments are not significantly different from the control at the $P = 0{\cdot}1$ level. Leaf mean fresh wts are not significantly different.

that MBC supplied early in the development of the leaf is effective in suppressing TMV RNA accumulation; MBC supplied late is not. This is a possible explanation of the differences in effect of MBC on TMV multiplication in lower and upper leaves (Figs 1 and 2).

A possible explanation of the antiviral activity of MBC

There are two types of mechanism by which MBC might inhibit TMV RNA accumulation in young leaves. One is indirect: by altering leaf metabolism in some way so as to make conditions unfavourable for viral multiplication. The other is that MBC might reduce viral RNA synthesis directly, for example by inhibiting the replicase. Other benzimidazole derivatives have been shown to inhibit certain animal virus RNA polymerases [*23*] and HeLa cell RNA polymerase II [*19*]. Direct inhibition of TMV RNA synthesis by MBC was tested experimentally, by examining rates of ^{32}P incorporation into TMV RNA in infected leaf discs newly exposed to MBC. The rate of incorporation of ^{32}P into 25 S rRNA was followed as an internal control, to detect any depression of RNA synthesis from non-specific causes.

Figure 5 shows that 2 μg/ml MBC had little or no effect on ^{32}P incorporation into TMV RNA or 25 S rRNA. Twenty μg/ml MBC reduced incorporation into TMV RNA by 40% and into 25 S rRNA by 60%. There was no further reduction in incorporation at 200 μg/ml, but MBC is not completely soluble at this concentration. Total uptake of ^{32}P by the discs was actually slightly stimulated by 2 or 20 μg/ml MBC, so the inhibition of incorporation of ^{32}P into TMV RNA and 25 S rRNA was likely to be an effect of MBC on RNA synthesis, not merely one on precursor uptake.

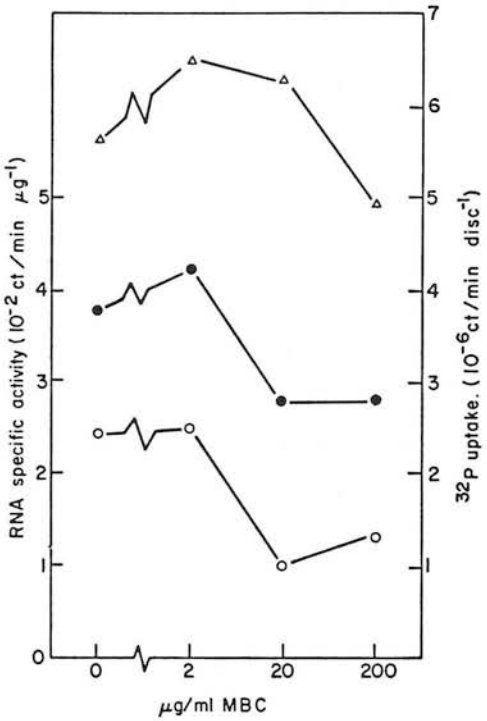

FIG. 5. Effects of MBC on RNA synthesis in TMV-infected leaf discs. Discs were punched from 9 cm long leaves of Samsun tobacco, 5 days after inoculation with TMV strain vulgare. (△—△) ^{32}P uptake; (●—●) ^{32}P incorporation into TMV RNA; (○—○) ^{32}P incorporation into 25 S rRNA.

From this experiment, there is evidence for a general depression of RNA synthesis at higher levels of MBC: there is no evidence for any selective inhibition of TMV RNA synthesis by MBC. This result is in contrast to the effect shown in Fig. 4, where young leaves exposed continually to MBC showed a significant inhibition of TMV RNA accumulation without any effect on 25 S rRNA content. The conclusion drawn from Fig. 5 is subject to the limitations on conclusions drawn from negative results; however, comparison of the effects shown by MBC in Figs 4 and 5 favours an inhibition of TMV RNA accumulation operating by indirect means. This is supported by the failure of MBC to inhibit TMV RNA accumulation in old leaves (Figs 1 to 3) and by the finding that MBC supplied early in the life of the leaf inhibits accumulation, while MBC supplied late, during the period of viral multiplication, does not (Table 2).

A possible mechanism for the proposed indirect suppression of TMV RNA accumulation by MBC is its known cytokinin activity [*21*, *24*]. Cytokinins have been reported to influence local lesion formation and infectivity [*1–5*, *8*]. Furthermore, cytokinins are known to delay leaf senescence [*22*]: it has been shown that the major accumulation of TMV RNA in leaves coincides with the onset of the senescent phase of leaf development [*10*]. Thus MBC, if applied to young leaves, may prevent viral multiplication by maintaining that leaf in some juvenile state unsuitable for

viral multiplication. This conclusion is consistent with the demonstrated failure of MBC to inhibit viral multiplication in old leaves (Figs 1 to 3) unless applied at extremely high concentration or very early in the life of the leaf (Table 2).

Long-term effects of MBC on growth and TMV RNA content

In the experiments reported in Figs 1 to 4, we followed TMV RNA content for up to 30 days after inoculation, by which time it had reached fairly stable levels in most treatments. The absence of any apparent direct viricidal effect of MBC, but the possibility that it may inhibit TMV multiplication by maintaining the leaf in some juvenile state, raised the question of long-term effects on growth and TMV RNA content. Accordingly, we examined plants 100 days after inoculation. These plants had completed vegetative growth and were well into the flowering phase.

In these plants, there was still a very striking difference in growth between MBC

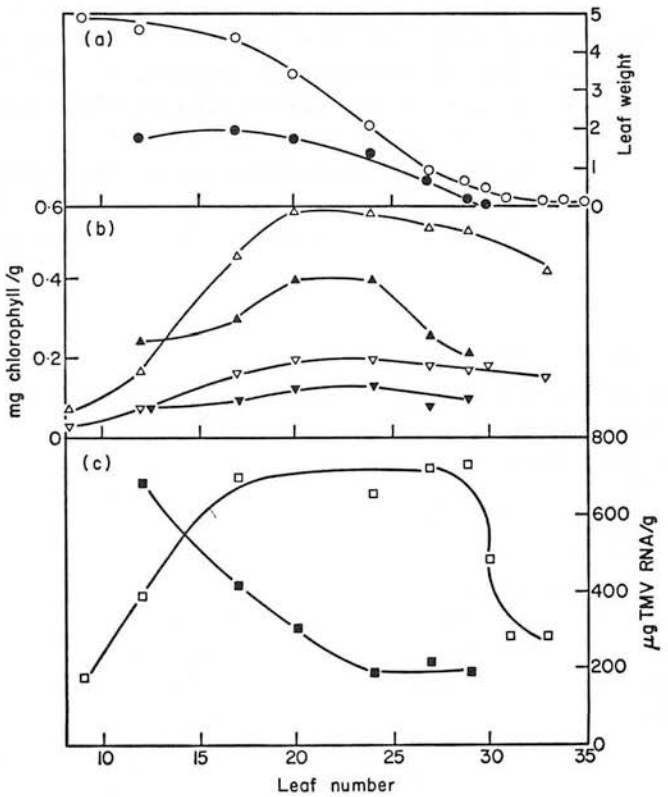

FIG. 6. Long-term effects of MBC on leaf growth, chlorophyll content and TMV RNA concentration in Samsun tobacco. Plants received 0·5 g MBC 9 and 2 days before inoculation of the highest expanded leaf with TMV strain Grapevine. Harvesting was 100 days after inoculation. Leaves were numbered upwards from the base. (a) Leaf fresh weight of MBC-treated (○—○) and control (●—●) plants. (b) Chlorophyll per g leaf. (△,▽) MBC-treated; (▲,▼) control plants; (△,▲) chlorophyll *a*; (▽,▼) chlorophyll *b*. (c) TMV RNA concentrations in control (■—■) and MBC-treated (□—□) plants.

and control treatments. MBC-treated plants had more leaves, of a greater fresh wt, than control plants (Fig. 6). The mean shoot weight of MBC-treated plants was over twice that of the control plants. The concentrations of chlorophylls *a* and *b* in treated plants remained higher than in control plants. Treated plants also showed much milder visible symptoms than control plants. Thus MBC treatments, 9 and 2 days before inoculation, had significant beneficial effects on plant growth; these effects persisted for a long time.

TMV RNA concentrations in MBC-treated and control plants 100 days after inoculation were very different. In the lowest remaining non-necrotic leaves, TMV/g was lower in leaves of treated plants than in those of control plants (Fig. 6). These leaves correspond to the upper leaves of Fig. 2, 30 days after inoculation. Thus, the inhibition of TMV RNA accumulation in these leaves by MBC treatment appears to be highly persistent.

In the higher leaves of MBC-treated plants, the concentration of TMV RNA at 100 days after inoculation was much higher than in untreated plants. There is therefore an apparent paradox: that treated plants have grown much better than control plants, but also contain much higher concentrations of virus. This outcome was possibly the consequence of two separate processes. During the vegetative growth phase of the plant, suppression of TMV RNA synthesis by MBC treatment (Fig. 4) prevented inhibition of leaf growth by the virus [*10*]: treated plants grew better than untreated control plants (Fig. 6). However, once the plants had finished the vegetative phase of growth and entered the flowering phase, the enhanced growth made as a result of MBC treatment was available as a source of substrates for viral synthesis.

TMV multiplication, symptoms and growth

The enhanced growth of TMV-infected plants as a result of MBC treatment is accompanied by a reduction in the severity of visible symptoms [*25*] and reduction in viral RNA accumulation in actively growing parts of the plant (Figs 2 and 4). From the results presented here, we can draw some conclusions about how leaf growth, symptoms and TMV multiplication are related.

One expression of visible symptoms is loss of chlorophyll. In young, infected leaves, MBC doses of more than 1 to 2 g per plant were required to maintain chlorophyll content at the same level as in healthy leaves, i.e. 1 mg/g (Fig. 4). Strong inhibition of viral multiplication was observed at doses as low as 0·1 g MBC per plant. Thus there is no direct correlation between the amount of virus synthesis and expression of visible symptoms as measured by loss of chlorophyll.

In young, infected leaves, the levels of MBC treatment which prevented inhibition of leaf growth by TMV infection were much lower than those required to prevent chlorophyll loss (Fig. 4). Thus the beneficial effect of MBC treatment on growth of infected leaves was probably not brought about by prevention of chlorophyll loss and any resulting reduction in photosynthetic capacity.

Viral RNA accumulation was inhibited by levels of MBC treatment similar to those preventing inhibition of leaf growth (Fig. 4). TMV reaches very high concentrations in tobacco leaves. Its multiplication requires the activity of a large

proportion of the host nucleic acid and protein synthesizing capacity [*10*, *11*]. Thus TMV multiplication is likely to act as a competitive inhibitor of host growth. We suggest that the enhanced growth of MBC-treated infected plants compared to control plants (Figs 4 and 6) may result from the indirect inhibition of TMV multiplication by MBC treatment during the active growth phase of the plant.

MBC at high levels inhibits growth of the host. From our data we can compare the doses required to inhibit viral multiplication and host growth. In old leaves treated late in their development with MBC, the doses inhibiting viral multiplication and host growth were similar (Fig. 2). From the standpoint of preventing viral inhibition of leaf growth by MBC, this is unimportant, as these leaves had partly completed growth in any case. More significant is the finding that in young, actively growing leaves, the MBC dose required to inhibit leaf growth was 50 to 100 times the dose required to prevent viral multiplication and viral inhibition of leaf growth (Fig. 4). MBC thus displays low phytotoxicity and permits significantly improved growth and appearance [*25*] of the host. One probable drawback to its use as a chemotherapeutic agent is that treated plants do eventually contain more virus than infected controls (Fig. 6; [*2*]). This might increase the rate of spread of virus to other plants. Possible deleterious effects of MBC treatment on leaf smoking quality and toxicity have also not been investigated.

We thank our colleagues for useful discussions, and Su Loughlin and Paul Tomlinson for technical assistance.

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Printed in Great Britain by John Wright and Sons Ltd. at The Stonebridge Press, Bristol BS4 5NU

J. gen. Virol. (1979), **43**, 131–141
Printed in Great Britain

Acquired Systemic Susceptibility to Infection by Tobacco Mosaic Virus in *Nicotiana glutinosa* L.

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(*Accepted* 6 *October* 1978)

SUMMARY

In *Nicotiana glutinosa* L. formation of local lesions on lower leaves inoculated with tobacco mosaic virus increased the susceptibility of the upper leaves to infection in a subsequent inoculation. The increase in susceptibility was detected as an increase of up to 3·5-fold in the number of lesions produced on the upper leaf and a corresponding increase in the amount of virus RNA synthesized. The concentration of endogenous abscisic acid in the upper leaves was negatively correlated with susceptibility to infection. This acquired systemic susceptibility to infection in *N. glutinosa* is in direct contrast to the acquired systemic resistance to infection reported to occur in hypersensitive varieties of *Nicotiana tabacum* under similar conditions. Mechanisms which might be involved in the acquisition of systemic resistance or susceptibility are discussed.

INTRODUCTION

The phenomenon of acquired systemic resistance has been extensively described in plants reacting to virus infection by formation of necrotic local lesions. If lower leaves are inoculated and develop lesions, the upper leaves become to some extent resistant to a subsequent challenge inoculation. This resistance is recorded as a reduction in lesion size, lesion number or both. The effect has been described in several host-virus combinations (Gilpatrick & Weintraub, 1952; Ross, 1961, 1964; Loebenstein, 1963; Nagaich & Singh, 1970; Kassanis *et al.* 1974). However, most studies have dealt with acquired resistance to tobacco mosaic virus (TMV) in varieties of *Nicotiana tabacum* L. such as Xanthi-nc (Kassanis & White, 1974, 1975) and Samsun NN (Ross, 1966; van Loon & van Kammen, 1970) containing the N gene (Holmes, 1938) for local lesion formation. It has been suggested that alteration in host protein synthesis (Kassanis *et al.* 1974; van Loon & van Kammen, 1970) or growth regulator metabolism (Balazs *et al.* 1977) may be involved in the acquisition of systemic resistance.

We have studied the systemic effects of local lesion formation in *Nicotiana glutinosa* L., a species which shows the local lesion reaction to TMV, and is the original source of the N gene (Holmes, 1938). In contrast to the acquired systemic resistance found in hypersensitive *N. tabacum*, we find that local lesion formation in the lower leaves of *N. glutinosa* increases the susceptibility of the upper leaves to infection in a subsequent inoculation.

Application of abscisic acid (ABA) to leaves of hypersensitive tobacco has been shown to increase the number of lesions formed as a result of subsequent inoculation with TMV (Balazs *et al.* 1973). In this paper we show that at the time of the challenge inoculation, the

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level of endogenous ABA in *N. glutinosa* leaves showing acquired systemic susceptibility was lower than in control leaves. The implications of these results for the mechanisms involved in acquired systemic resistance and susceptibility are discussed.

METHODS

Host plants and viruses. Nicotania glutinosa L. plants were grown in John Innes no. 2 compost in 12·5 cm diam. pots. The plants were kept in a glasshouse under natural lighting. The temperature was maintained at 16 °C at night; during the day it rose to 20 to 25 °C depending on the season.

Tobacco mosaic virus strains *vulgare* and *flavum* (originally obtained from the Max Planck Institut für Biologie, Abteilung Melchers, Tübingen, West Germany) were multiplied in the systemic host *Nicotiana tabacum* cv. Samsun. Infected leaf material was stored at −20 °C. Inocula were prepared by grinding infected leaf material in 50 mM-sodium phosphate buffer, pH 7·0, using a pestle and mortar. The homogenate was filtered through muslin and diluted with phosphate buffer as required.

N. glutinosa plants were given a first inoculation when 15 to 20 cm tall, with five to eight fully-expanded leaves. Three lower expanded leaves on each plant were lightly dusted with 400-mesh Carborundum and inoculated by rubbing with virus suspension. Control plants were sham-inoculated by rubbing with sterile phosphate buffer, or with ground, frozen healthy leaf material at the same final dilution in phosphate buffer as the virus inoculum. Leaves were washed with water after inoculation.

Seven to ten days after the primary inoculation, the lesions on the inoculated leaves were counted. Three upper expanded leaves on each plant were dusted with Carborundum and inoculated with TMV suspension (the 'challenge' inoculation). Lesions on the upper inoculated leaves were counted 7 days later. Statistical tests of differences between lesion numbers in treatments were by *t*-test, using transformations for lesion numbers with small or very low means described by Kleczkowski (1949, 1955).

Lesion diam. were measured on the upper, challenge-inoculated leaves 7 days after inoculation, using a stereoscopic microscope with × 50 magnification. One randomly-chosen diam. of each of 7 lesions on each of 18 leaves was measured, giving a total sample of 126 lesions for each treatment.

TMV accumulation in challenge-inoculated upper leaves was measured 7 days after inoculation, using a stereoscopic microscope with × 50 magnification. One randomly-chosen Fraser & Whenham (1978).

Most experiments were done with intact plants. However, in some experiments indicated below, the plants were decapitated up to one week before primary inoculation.

Abscisic acid determination. The endogenous abscisic acid (ABA) content of upper leaves of *Nicotiana glutinosa* was measured at the time of the challenge inoculation, 7 days after primary inoculation of the lower leaves. For each treatment, seven plants were used for ABA determination; seven similarly-treated plants received the challenge inoculation.

Ten to 20 g fresh weight of upper leaves were homogenized in 150 ml ice-cold 80% (v/v) methanol using an Ultra-Turrax homogenizer. DL-*cis-trans*-2-^{14}C-abscisic acid (5 nCi; sp. act. 11·1 mCi/mmol; Radiochemical Centre, Amersham, U.K.) was added to the homogenate to allow estimation of the percentage recovery of ABA after purification and to facilitate location of ABA on chromatograms.

ABA in the homogenate was partially purified by successive acid/base partitioning between water and dichloromethane (Zabadal, 1974). ABA was further purified by chro-

matography on 3MM paper developed with isopropanol/ammonia/water (8:1:1, v/v). ABA was located on the chromatogram in a spark chamber radiochromatogram scanner (Birchover Instruments Ltd) and eluted with 80% methanol. After methylation with ethereal diazomethane (Schlenk & Gillerman, 1960), the ABA was rechromatographed on thin layer plates of silica gel 60 F_{254} (Merck) multiply-developed with hexane/ethyl acetate (1:1, v/v).

ABA was located on the chromatogram in the spark chamber. The fraction containing the ABA was scraped off the plate and ABA was eluted with ether. The extract was dried, then taken up in 50 μl ethyl acetate. Samples of 5 μl were mixed with 10 ml scintillator [60%, v/v, toluene; 40%, v/v, 2-methoxyethanol, containing 5 g/l 2(4′-*t*-butylphenyl)-5-(4″-biphenylyl)-1,3,4,-oxadiazole]. Disintegrations per minute of the ^{14}C-ABA in the extract, hence the percentage efficiency of the ABA extraction, were measured by scintillation counting. The counting efficiency of each sample was determined by internal standardization, by addition of a known activity of ^{14}C-toluene (Radiochemical Centre, Amersham).

Total ABA content of the extract was measured by gas chromatography. Samples of 5 μl of the extract were chromatographed on a column containing 5% SE-30 coated on 80- to 100-mesh Chromosorb W AW-DCMS. The column temperature was 200 °C; the carrier gas was oxygen-free nitrogen at a flow rate of 40 ml/min. The chromatograph was equipped with a flame-ionisation detector. *Cis*- and *trans*-methyl abscisate were identified on chromatograms by co-chromatography with authentic *cis*- and *trans*-methyl abscisate (ABA from Sigma Chemical Co., methylated as above) and by combined gas chromatography-mass spectrometry.

ABA was measured by the peak area of *cis*- plus *trans*-methyl abscisate on the gas chromatograph trace, calibrated by chromatography of known amounts of pure *cis*- and *trans*-ABA methylated as above, and corrected to 100% recovery using the ^{14}C-ABA recovery measurements.

RESULTS

Acquired systemic susceptibility

Fig. 1 shows that when *Nicotiana glutinosa* plants were previously inoculated on the lower leaves, the number of lesions produced on subsequent inoculation of the upper leaves was greater than in control plants. Primary inoculation of the lower leaves thus made the upper leaves in some way more susceptible to infection by TMV. Khuruna & Hidaka (1977) have shown that the non-infected leaves of TMV-inoculated *N. glutinosa* contain an extractable factor which, if mixed with TMV suspension, will increase the number of lesions formed by that inoculum when applied to a fresh *N. glutinosa* plant. We do not know at present whether our *in vivo* acquired systemic susceptibility involves the operation of such a factor.

The degree of acquired systemic susceptibility produced depended on the lesion density on the lower, previously inoculated leaves. Generally, the more concentrated the virus suspension used to inoculate the lower leaves, the greater the increase in susceptibility to infection of the upper leaves (Fig. 1). If the mean number of lesions per upper challenged leaf is plotted against mean number of lesions per lower inoculated leaf for individual plants and the data are then grouped on the basis of lower leaf lesion numbers, then an approximately linear relationship is obtained between log challenged-leaf lesions and log lower-leaf lesions (Fig. 2).

In the experiment shown in Fig. 1 and 2, the range of dilutions of inoculum used for the lower leaves was sufficiently wide to produce all possible responses in the lower leaves.

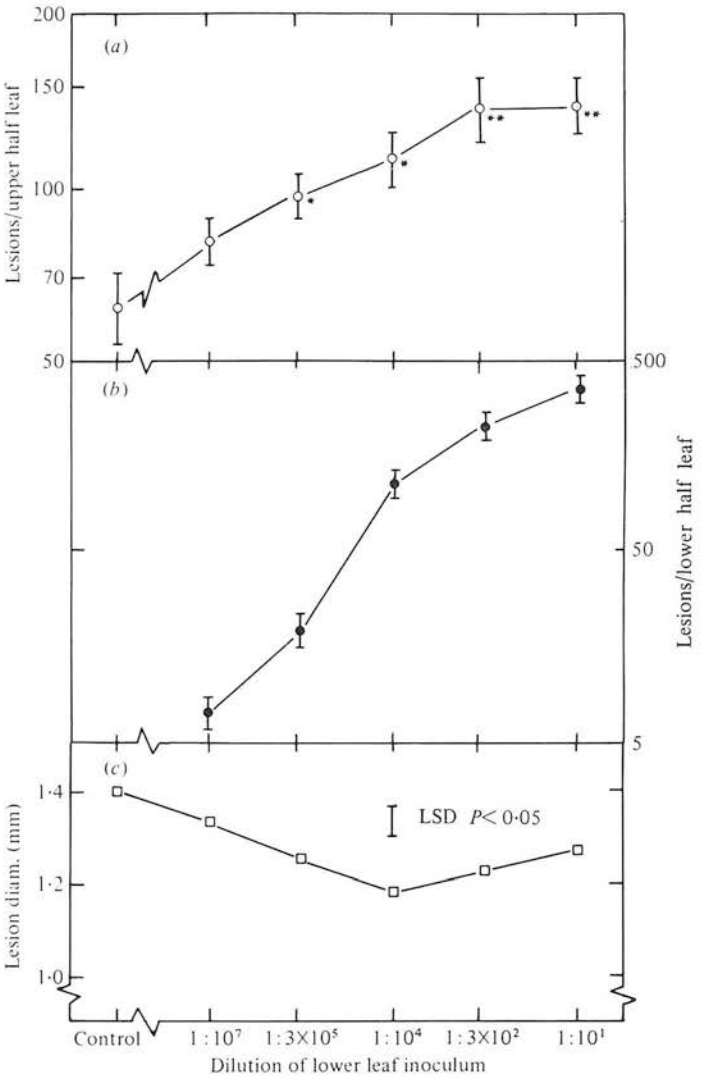

Fig. 1. Acquired systemic susceptibility to infection by TMV in leaves of *Nicotiana glutinosa*. Three lower leaves on each plant were inoculated with sterile phosphate buffer (control) or with various dilutions of TMV strain *vulgare*; (*b*) shows the mean numbers of lesions which developed in each treatment. Seven days after the primary inoculation, three upper leaves on each plant were challenge-inoculated with TMV *vulgare* in sap diluted $1:10^4$. Lesion numbers on the upper leaves (*a*) were counted 7 days later. (*) and (**) indicate values significantly different from the control at $P = 0.01$ and $P = 0.001$, respectively. Data shown in (*a*) and (*b*) are means $\pm$ s.e. mean; (*c*) shows mean lesion diam. on the challenge-inoculated leaves. The vertical bar is the least significant difference (LSD) between treatments at $P = 0.05$.

The most dilute inocula produced a few lesions on most leaves inoculated, but failed to induce any lesions on some. The most concentrated inocula produced complete necrotic collapse of all inoculated lower leaves within 7 days of inoculation and thus stimulated the maximum possible expression of the hypersensitive reaction.

No lesions were found on the upper leaves of any plants when these leaves received no challenge inoculation, or when they were sham-inoculated with sterile phosphate buffer.

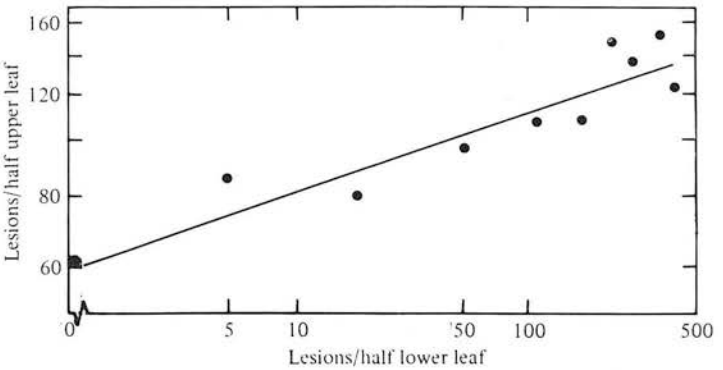

Fig. 2. Acquired systemic susceptibility to infection by TMV in upper leaves of *Nicotiana glutinosa*: the relation between lesion numbers on the lower and upper leaves. For individual plants, the mean number of lesions on the upper, challenge-inoculated leaves was plotted against the mean number on the lower, previously inoculated leaves. The data were then grouped on the basis of lower leaf lesion numbers. The categories for grouping were controls (no lower leaf lesions); plants with lesions on the lower leaves were grouped in 4's in ascending order of lesion number.

Table 1. *TMV RNA concentration and number of local lesions formed on challenge-inoculated upper leaves**

Inoculum used for lower leaves	TMV RNA (μg/g upper leaf)	Lesions per half upper leaf
TMV	$10{\cdot}1 \pm 0{\cdot}8$	1222 ± 76
Sterile phosphate buffer (control)	$3{\cdot}1 \pm 0{\cdot}5$	331 ± 40

* Plants were inoculated on three lower leaves with TMV strain *flavum* in sap diluted $1:10^3$, or with sterile phosphate buffer (control). Seven days later the three upper leaves of all plants were inoculated with TMV strain *flavum* in sap diluted $1:10^2$. Lesion numbers and TMV RNA contents were measured 7 days after the second inoculation. All values are means $\pm$ s.e. mean. The differences between the treatments are significant at $P = 0{\cdot}01$.

Thus the increase in number of lesions formed on the upper leaves of plants previously inoculated on the lower leaves was not the result of systemic spread of virus from the lower inoculated leaves.

Table 1 shows amounts of TMV RNA found in upper inoculated leaves of plants which were showing acquired systemic susceptibility and control plants. Because of the restriction of virus multiplication by local lesion formation, TMV will only multiply to very low concentrations in leaves of *N. glutinosa*. It was therefore necessary to use a high concentration of inoculum for the upper, challenge-inoculated leaves in this experiment, to produce high lesion numbers and thus comparatively high and measurable amounts of TMV RNA. It is clear that considerably more TMV RNA was present in leaves showing acquired systemic susceptibility than in the control leaves. The increased number of lesions found on leaves of plants made more susceptible to infection by a previous inoculation was thus paralleled by a similar increase in virus accumulation.

This result, plus the absence of any lesion formation on uninoculated upper leaves, allows us to exclude the possibility that the increased number of lesions formed on upper leaves of plants after primary inoculation of the lower leaves was due to the formation of 'non-viral lesions', such as have been reported to form on uninoculated parts of *N. glutinosa* plants kept under continuous light (Shimomura & Ohashi, 1975).

Table 2. *Acquired systemic susceptibility in* Nicotiana glutinosa *at various times of year**

Date of challenge inoculation	TMV strain	Lesions per half leaf		Degree of acquired systemic susceptibility
		Control plants	Previously inoculated plants	
29 Sept.	*flavum*	331 ± 40	1222 ± 76	3·69
29 Sept.	*vulgare*	338 ± 50	1092 ± 59	3·23
10 Feb.	*flavum*	3·5 ± 0·7	9·4 ± 1·7	2·68
28 Feb.	*flavum*	13·8 ± 1·6	38·7 ± 6·6	2·80
28 Mar.	*flavum*	68 ± 15	118 ± 30	1·76
1 June	*vulgare*	62 ± 9	139 ± 17	2·23
16 June	*vulgare*	31 ± 4	44 ± 5	1·40 NS
16 June	*vulgare*	40 ± 4	68 ± 9	1·72

* Seven days before challenge inoculation of the upper leaves, plants were inoculated on three lower leaves with sterile phosphate buffer (control plants) or with the TMV strain stated (previously inoculated plants). All plants were challenge inoculated on three upper leaves. The degree of acquired susceptibility is the mean number of lesions per upper half leaf on previously inoculated plants divided by the lesion number on leaves of control plants. Except where marked NS, differences between treatments are significant at $P = 0{\cdot}05$. All values are means ± s.e. mean.

Reproducibility of acquired systemic susceptibility

To determine whether the acquired systemic susceptibility in *N. glutinosa* was a reproducible phenomenon, we examined its occurrence under various experimental and environmental conditions. Table 2 is a summary of several experiments. It is clear that acquired systemic susceptibility could be observed at all seasons, and therefore over a wide range of natural lighting conditions. The effect occurred both with a common (*vulgare*) strain of TMV and with the yellow strain *flavum*.

The increase in lesion number on the upper leaves of plants showing acquired systemic susceptibility ranged from 1·4- to 3·7-fold. In no case did we observe any significant reduction in lesion number on the upper leaves as a result of prior inoculation of the lower leaves.

The numbers of lesions produced on the challenge-inoculated leaves varied considerably in different experiments, as a consequence of the different dilutions of challenge inocula used. There was some tendency for the highest levels of acquired systemic susceptibility to be associated with the highest absolute lesion numbers on the challenged leaves, but in some experiments high levels of acquired susceptibility were found in upper leaves with low lesion numbers (Table 2).

Acquired systemic susceptibility was observed equally in young plants and in old plants well into the flowering phase. Decapitation reduced the absolute numbers of lesions in all treatments, but also reduced the relative increase in susceptibility caused by previous inoculation of the lower leaves (Table 3). However, decapitated plants still showed statistically significant acquired systemic susceptibility.

Sham-inoculating the lower leaves of control plants with diluted, healthy leaf material homogenate rather than sterile phosphate buffer made no difference to the level of acquired susceptibility obtained. The susceptibility induced is thus unlikely to have been associated with any effect of a normal plant component present in the homogenate of infected leaf used as primary inoculum for the virus treatments.

When *Nicotiana tabacum* plants carrying the N gene for resistance to TMV (Holmes, 1938) were grown in the same glasshouse, the classical acquired systemic resistance was observed. Plants on which three lower leaves had been previously inoculated with TMV

Table 3. *Effects of decapitation on acquired systemic susceptibility in* Nicotiana glutinosa*

Treatment of plants	Lesion number per challenged upper half leaf		Degree of acquired systemic susceptibility
	Control plants	Previously inoculated plants	
Intact	62 ± 9	113 ± 13	1·82
Decapitated	49 ± 5	64 ± 6	1·31 NS
Intact	62 ± 9	139 ± 17	2·23
Decapitated	24 ± 5	47 ± 8	1·96

* Two experiments are shown. For the first, plants were decapitated immediately before the primary inoculation. For the second, plants were decapitated one week before the primary inoculation. Three lower leaves on each plant were inoculated with sterile phosphate buffer (control plants) or with TMV strain *vulgare* (previously inoculated plants). The degree of acquired systemic susceptibility induced by primary inoculation of the lower leaves was calculated as explained in the legend to Table 2. With the exception of the value marked NS, differences between treatments are significant at $P = 0{\cdot}05$.

showed approx. 50% reduction in mean lesion diam. and lesion number on the challenge-inoculated upper leaves compared to controls (our unpublished results). Thus the acquired systemic susceptibility displayed by *N. glutinosa* appears to be a feature of the host species, rather than the environment.

Acquired systemic susceptibility and lesion size

Fig. 1 shows mean lesion diam. in upper, challenge-inoculated leaves showing various degrees of acquired systemic susceptibility. It is clear that increase in lesion numbers on the leaves of plants showing increased susceptibility was associated with a small, though statistically significant reduction in mean lesion diam. The largest decrease in mean lesion diam. was 16% of the control and was obtained when the lower leaves had been inoculated with an inoculum rather more dilute than that required to induce the highest level of acquired susceptibility.

Endogenous abscisic acid and acquired systemic susceptibility

Balazs *et al.* (1973) have shown that when tobacco leaves are treated with high concentrations of exogenous ABA, their susceptibility to infection by tobacco mosaic virus increases, resulting in an increased number of lesions on the treated leaves. To determine whether the acquired systemic susceptibility in *N. glutinosa* was related in any way to endogenous levels of ABA, we measured endogenous ABA concentrations at the time of the challenge inoculation. Plants were previously inoculated on the lower leaves with different concentrations of virus suspension, to produce a range of lesion densities, or with sterile phosphate buffer as controls. At the time of the challenge inoculation, plants in each treatment were split into two groups. Upper leaves of one group were challenge inoculated and used to determine the level of acquired systemic susceptibility induced by the treatment. Upper leaves of the other group were used for ABA determinations.

The concentration of ABA in upper leaves of control (sham-inoculated) plants varied from 25 to 65 ng/g fresh weight, depending on the experiment and environmental conditions. Inoculation of the lower leaves resulted in a reduction in the concentration of ABA in the upper leaves at the time of the challenge inoculation 7 days later. Fig. 3 shows the collected results of replicate experiments. All ABA values are expressed as percentages of the ABA

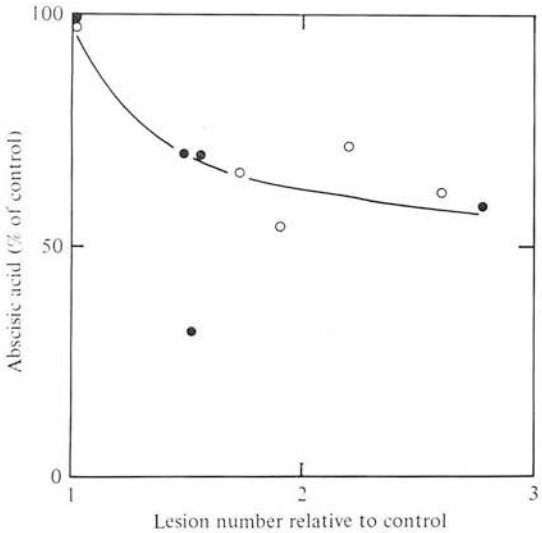

Fig. 3. Endogenous abscisic acid concentrations in *Nicotiana glutinosa* leaves showing acquired systemic susceptibility. Data from two separate experiments are shown (○) and (●). ABA levels were measured in the upper leaves 7 days after sham-inoculation of the lower leaves (controls) or after inoculation of the lower leaves with various concentrations of TMV strain *flavum*. Upper leaves on plants treated in parallel were challenge-inoculated at the same time as the ABA determination. Lesions that developed on the challenged leaves were counted, and the increase in lesion number on leaves showing acquired susceptibility relative to the controls was calculated.

concentration in the sham-inoculated control for that experiment. Generally, the higher the level of acquired systemic susceptibility recorded in the upper leaves, the lower their ABA concentration (relative to the control) at the time of the challenge inoculation. Thus we find a negative correlation between endogenous ABA concentration and susceptibility to infection by TMV, in contrast to the result of Balazs *et al.* (1973). However, in considering the differences between the two sets of results, it should be remembered that Balazs *et al.* (1973) used ABA concentrations vastly in excess of those occurring naturally.

DISCUSSION

In this paper, we have shown that inoculation of the lower leaves of *Nicotiana glutinosa* with TMV alters the response of the upper leaves to a subsequent inoculation with TMV. This alteration takes two forms: the lesions formed on the challenged upper leaves are slightly reduced in size, and are considerably more numerous, than on similarly treated leaves of control plants. It is likely that separate mechanisms underly these two changes in response induced by the initial local lesion formation on the lower leaves.

Ross (1966) found that prior inoculation of Samsun NN tobacco with TMV resulted in a reduction of up to 80% in the size of lesions formed on subsequent challenge-inoculation of previously uninoculated parts of the plant. This reduction in size has been interpreted as evidence for systemic acquired resistance. Balazs *et al.* (1977) however have suggested that the reduction in lesion size is a consequence of failure of tissue to turn necrotic, not a reduction in the amount of virus multiplication in the challenge-inoculated leaf. In our experiments, the maximum reduction in lesion diam. was only 16%. Clearly, any resistance

mechanism which operates by reducing the size of lesions in the challenged leaf has very much less activity in *N. glutinosa* than in hypersensitive *Nicotiana tabacum*.

Many reports of acquired systemic resistance in hypersensitive *Nicotiana tabacum* show that the number of lesions formed on the challenged leaf is reduced as a result of prior inoculation of the lower leaves with TMV (Ross, 1966; Kassanis *et al.* 1974). Treating the challenged or lower leaves with polyanions such as polyacrylic acid before challenge inoculation also reduces the number of lesions formed (Gianinazzi & Kassanis, 1974; Kassanis & White, 1974, 1975; Cassells *et al.* 1978). Our results showing acquired systemic susceptibility in *N. glutinosa* are thus the direct opposite of the acquired systemic resistance found in hypersensitive varieties of *N. tabacum*. Our results show that the presence of the N gene and its activity in local lesion formation do not inevitably lead to acquired resistance as the overriding response. This raises the question of what mechanisms are involved in the acquisition of systemic susceptibility or resistance.

Two classes of mechanism may be proposed. For acquired systemic resistance, it has been suggested that a specific antiviral mechanism operates against infection or virus multiplication, to reduce lesion number or size (Ross, 1966; Kassanis *et al.* 1974). Certain new proteins ('b' proteins) become detectable in the resistant leaves after primary virus inoculation or polyacrylic acid treatment (van Loon & van Kammen, 1970; Kassanis *et al.* 1974; Antoniw & Pierpoint, 1978). It has been suggested that these 'b' proteins may be involved in the resistance reaction, perhaps in a manner analagous to interferon in animals (Gianinazzi & Kassanis, 1974).

Clearly, our finding of acquired systemic susceptibility cannot be explained on the basis of any such specific resistance mechanism. Acquired susceptibility does not, however, in itself exclude the possibility that an interferon-like mechanism might operate in *N. glutinosa*: the resistance mechanism could be obscured by a separate mechanism stimulating lesion formation. An interferon-like mechanism could be responsible for the slight reduction in lesion diameter observed in leaves showing increased susceptibility as measured by increased lesion numbers (Fig. 1). van Loon & van Kammen (1970) did find new proteins in non-infected parts of *N. glutinosa* after lesion formation; these proteins were in some ways similar to the 'b' proteins of *N. tabacum*. However, 'b' proteins have not yet been shown to have any direct antiviral action (Antoniw & Pierpoint, 1978), and van Loon (1975) was able to induce acquired resistance in Samsun NN tobacco with mercuric chloride without the occurrence of 'b' proteins. The existence and function of an interferon-like antiviral mechanism in the response of the plant to a second inoculation thus remain to be established. The acquired systemic susceptibility shown by *N. glutinosa* suggests that induction of an antiviral mechanism is unlikely to give a complete explanation of the altered response to the challenge inoculation.

An alternative model may offer an explanation of both acquired systemic resistance and susceptibility. Increases or decreases in lesion numbers might arise from changes induced in the metabolic or physiological state of the host which alter the susceptibility of sites on the challenged leaf to infection or lesion development. Cassels *et al.* (1978) have shown in Xanthi-nc tobacco that the resistance to TMV induced by polyacrylic acid is associated with increased leaf water stress. They suggest that the loss of turgidity may reduce the susceptibility of surface sites to infection by TMV. Experimental support for this hypothesis comes from their observation that treatment of leaves with an anti-transpirant abolishes the polyacrylic acid-induced resistance (Cassells *et al.* 1978).

Our results with *N. glutinosa* are also consistent with the idea that alterations in leaf water status affect susceptibility to infection. Abscisic acid levels are known to increase with

increasing leaf water stress (Zabadal, 1974; Beardsell & Cohen, 1975). We consistently found that leaves with acquired susceptibility to infection had lower ABA concentrations than control leaves (Fig. 3), suggesting that such leaves had lower water stress and greater turgidity than control leaves. This may explain their enhanced susceptibility to infection.

It remains to be seen how local lesion formation on the lower leaves of tobacco plants can alter the water relations of the upper leaves. However, cytokinins (Livné & Vaadia, 1965; Biddington & Thomas, 1978) and abscisic acid (Cummins *et al.* 1971; Zabadal, 1974) are known to affect transpiration and stomatal opening. The demonstration that formation of local lesions on lower leaves can alter the ABA concentrations (Fig. 3) and cytokinin concentrations (Balazs *et al.* 1977) in uninfected upper leaves provides the basis of a possible mechanism.

We thank the Mass Spectrometry Group, Food Research Institute, Norwich, for GC-MS authentication of our ABA preparations. We thank Dr Alan Cassells and Dr John Antoniw for discussions and for allowing us access to their results prior to publication.

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(*Received* 28 *July* 1978)

Reprinted from *Physiological Plant Pathology* (1979) **14**, 383–394

Systemic consequences of the local lesion reaction to tobacco mosaic virus in a tobacco variety lacking the *N* gene for hypersensitivity

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(*Accepted for publication December 1978*)

White Burley tobacco is a differential host for tobacco mosaic virus. The virus strain *vulgare* gives a systemic infection. In contrast, the strain *flavum* is restricted to necrotic local lesions which form round the point of infection. When plants formed local lesions on the lower leaves after inoculation with *flavum*, the lesions formed on the upper leaves in response to a subsequent challenge inoculation with *flavum* were smaller and fewer than those formed on comparable leaves of plants which had not received the first inoculation. The differential host therefore showed systemic consequences of the initial local lesion reaction similar to the "acquired systemic resistance" described by other authors for tobacco varieties containing the *N* gene, which causes a local lesion reaction against all strains of tobacco mosaic virus.

Multiplication of tobacco mosaic virus in the challenge-inoculated leaves was followed by measuring the accumulation of viral RNA. It is shown that formation of *flavum* lesions on the lower leaves did not reduce the accumulation of *flavum* or the systemic strain *vulgare* when the upper leaves were challenge-inoculated with these strains. These results imply that "systemic acquired resistance" as measured by reduction in lesion size and number is not resistance as measured by inhibition of viral multiplication. The mechanisms involved in restricting lesion size and number in the "resistant" leaves are discussed.

INTRODUCTION

The *N* gene from *Nicotiana glutinosa* L. has been introduced into many varieties of *Nicotiana tabacum* L. [*11*, *23*]. When tobacco plants containing this gene are inoculated with tobacco mosaic virus (TMV), the virus is normally restricted to the necrotic local lesions which form around the sites of infection: the virus does not spread systematically. Development of local lesions after inoculation may alter the response of uninfected parts of the plant to a subsequent inoculation. For example, lesion formation on the lower leaves of the *N* gene tobacco varieties Samsun NN and Xanthi-nc can reduce the number [*12*, *20*, *21*, *26*] and size [*2*, *15*, *20*, *22*, *27*] of lesions formed when the upper leaves are subsequently inoculated. This phenomenon has been referred to as "acquired systemic resistance" [*15*, *21*]. It has been suggested that alterations in host protein synthesis [*12*, *26*, *29*], water relations [*7*] or hormone metabolism [*1*, *2*] may be involved.

Certain varieties of tobacco which do not contain the *N* gene will act as differential hosts for TMV [*18*, *25*]. These varieties will form necrotic local lesions with

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some strains of TMV, whereas other strains elicit no hypersensitive response, but spread systemically. The primary aim of this study was to determine whether systemic consequences of local lesion formation, such as "acquired systemic resistance", occur when the hypersensitive reaction is determined by the nature of the virus in such a differential host. It is shown that size and number of lesions formed on the upper leaves of a differential host are lower if the plants had formed lesions on the lower leaves previously.

In tobacco varieties containing the *N* gene, there has been some doubt about whether the "acquired systemic resistance" actually involves any inhibition of virus multiplication in the inoculated "resistant" leaves. For example, Balazs *et al.* [*1*, *2*] have suggested that the induced resistance in Xanthi-nc tobacco, expressed as a reduction in lesion size and number, may simply reflect a failure of lesions to turn necrotic, rather than an inhibition of virus multiplication. This interesting conclusion was, however, based on estimates of TMV content by infectivity assay, a technique unlikely to detect small differences in TMV concentration between treatments [*3*]. Others [*21*, *26*] have concluded that "resistant", challenge-inoculated leaves do contain less virus than control leaves.

The second aim of this work therefore was to compare the accumulation of TMV in leaves showing the "acquired systemic resistance" effects of fewer and smaller lesions, with TMV accumulation in control leaves, by a technique which is capable of detecting small differences in TMV content between treatments. Again use was made of the differential host. This permitted measurement of the consequences of local lesion formation on the lower leaves for the accumulation of either the local lesion or the systemic strains of TMV when subsequently these were inoculated on the upper leaves. The systemic strain multiples to a much greater extent than the local lesion strain, and thus provides a different and perhaps greater test of any induced resistance mechanism.

MATERIALS AND METHODS

Plants and viruses

The differential host was *Nicotiana tabacum* L. var. White Burley. In this, the TMV strain *vulgare* becomes systemic; the TMV strain *flavum* [*17*] is localized in necrotic lesions. *N. tabacum* var. Burley S_3, which does contain the *N* gene, forms necrotic local lesions with both virus strains, and was used for comparisons with the differential host.

Plants were grown in John Innes no. 2 compost in 12·5 cm diameter pots. The plants were kept in a glasshouse under natural lighting conditions. The temperature was maintained at 16 °C at night and at 20 to 25 °C during the day, depending on the season.

The two virus strains were multiplied in Samsun tobacco, in which both are systemic. Infected leaves were stored frozen at −20 °C. Inocula were prepared by grinding stored leaf with 50 mM sodium phosphate buffer, pH 7·0, using a pestle and mortar. The inocula were diluted with phosphate buffer to produce the final lesion density required.

Inoculation

White Burley and Burley S_3 plants with four to six expanded leaves were dusted lightly with 400-mesh carborundum on two middle expanded leaves. These were inoculated by hand rubbing with virus suspensions at appropriate dilution, then rinsing with water. The strain of virus used is given in Results for each experiment. Control plants were sham-inoculated with sterile phosphate buffer. This inoculation of the lower leaves is referred to as the "primary" inoculation. At least seven plants were used for each treatment.

Ten days after primary inoculation, the lesions on the inoculated leaves were counted. The two highest expanded leaves on each plant (including sham-inoculated controls) were then inoculated with *flavum* or *vulgare*, as detailed below. This inoculation of the upper leaves is referred to as the "challenge" inoculation. The same dilution of challenge inoculum was used for all treatments in an experiment.

Lesions on the challenge-inoculated leaves were counted 10 to 15 days after inoculation. Lesion size was estimated by measuring one randomly chosen diameter of 100 to 200 lesions for each treatment, using a stereoscopic microscope with a magnification of $\times 50$. Lesions were measured on at least 10 leaves from 10 separate plants for each treatment. Leaf areas of the challenge-inoculated leaves were measured with a television leaf area monitor 10 days after challenge inoculation.

TMV multiplication

Samples of 1 g challenge-inoculated leaf were taken at various times after inoculation. Virus content of the samples was measured as TMV RNA. This was extracted, purified, separated by polyacrylamide gel electrophoresis and measured as explained elsewhere [*9*, *10*]. At least four plants were sampled for each treatment at each harvest.

Statistical analysis

Differences between treatments were analysed for significance by Student's *t*-test. Lesion numbers were transformed logarithmically as explained by Kleczkowski [*14*] before analysis.

RESULTS

Lesion size and number in challenge-inoculated leaves

Figure 1 shows the effects of local lesion formation on the lower leaves of *flavum*-infected White Burley on lesion formation on the challenge-inoculated upper leaves. The lower leaves were inoculated with a wide range of dilutions of virus: the response in these leaves varied from very few lesions at the greatest dilution to general necrotic collapse of up to 20% of the leaf area at the lowest dilution. Lesion numbers on the primarily inoculated leaves are presented in Fig. 1 as per unit leaf area, as at the lower inoculum dilutions the lesions per leaf were too numerous to count conveniently.

The upper leaves of all plants, including the controls which had been sham-inoculated on the lower leaves, were challenge-inoculated with the same dilution of

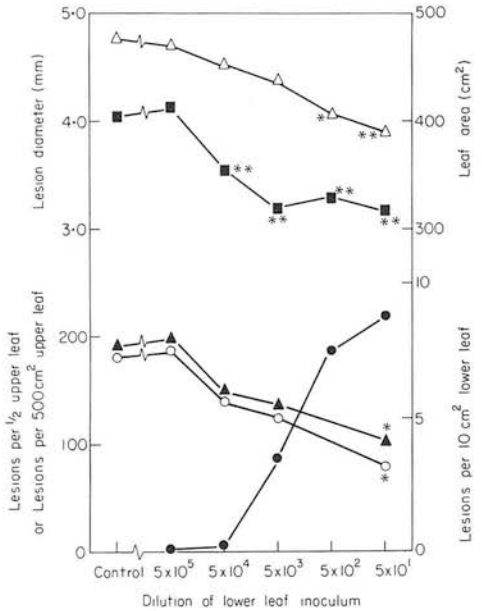

FIG. 1. Lesion numbers and diameters, and leaf areas, of TMV strain *flavum*-infected White Burley tobacco plants. Lower leaves were infected with sterile phosphate buffer (control) or with various dilutions of virus solution. (●) Numbers of lesions developing on the inoculated lower leaves. The upper leaves of all plants were challenge-inoculated with TMV strain *flavum* at a dilution of $1 : 5 \times 10^3$, 10 days after the primary inoculation. (■) Mean lesion diameter on upper leaves, measured 10 days after challenge-inoculation. (○) Number of lesions per half challenge-inoculated leaf. (△) Upper leaf area, measured 10 days after challenge-inoculation. (▲) Lesions per unit area of challenge-inoculated leaf. Values marked (*) and (**) are significantly different from the control value at $P = 0{\cdot}05$ and $P = 0{\cdot}01$ respectively.

flavum. Thus any differences in lesion size or number on the challenge-inoculated leaves were a consequence of the treatment applied to the primarily-infected lower leaves.

Several differences were apparent in challenge-inoculated leaves between treatments. The number of lesions formed on the upper leaves was lower when the lower leaves had been inoculated with virus than when the lower leaves had been sham-inoculated. The reduction in lesion number on the challenge-inoculated leaves increased with increasing density of lesions on the primarily-inoculated leaves (Fig. 1). The diameter of lesions on the challenge-inoculated leaves was also reduced in plants where the lower leaves had formed lesions. Again, the greater the density of lesions on the lower leaves, the greater the reduction in lesion diameter. Finally, the area of the challenge-inoculated leaves was reduced in plants with TMV-inoculated lower leaves; the reduction in area was greater when the lower leaves had high lesion densities. Measurements of the lengths of challenge-inoculated leaves at the time of challenge inoculation confirmed that the differences in size were established by this stage in the experiment. Reduction in leaf size was thus a consequence of the effects of the primary inoculation.

Smaller leaves might perhaps have fewer infectible sites than large leaves: the effects of lesions on the lower leaves on upper leaf area should thus be taken into account when measuring effects of lower leaf treatments on numbers of lesions formed on the challenge-inoculated upper leaves. Figure 1 shows that when the numbers of upper leaf lesions were expressed per unit leaf area, lesion density on the upper leaves was still inversely proportional to lesion density on the primarily-inoculated leaves. The reduction in lesion density was, however, less marked than the reduction in absolute lesion numbers per upper half leaf.

This experiment has been repeated at different times of year, with consequent differences in natural lighting conditions. In each case the reductions in lesion number, diameter and leaf area have been observed. On average, in treatments showing the maximum difference from the control, lesion number was reduced by 25 to 50%, lesion diameter was reduced by rather less (15 to 40%) and leaf area was least affected, being reduced by 10 to 25%.

In the different experiments, the absolute numbers of lesions formed on the challenge-inoculated leaves varied as a result of the different concentrations of challenge inoculum used. The range of mean lesion number was from 10 to 1000 per half challenge-inoculated leaf. There was no obvious correlation between absolute lesion number and degree of reduction in lesion size or number compared to the controls.

A differential host is, by definition, subject to systemic infection by some strains of virus. White Burley plants inoculated with *flavum* on the lower leaves frequently showed development of systemic symptoms on the very youngest upper leaves. These symptoms appeared in 25 to 50% of plants, generally within 10 to 30 days of the primary inoculation. They were probably caused by naturally occurring mutants in the inoculum used for the lower leaves, or by mutants arising during the limited virus multiplication in the lower leaves. The nature of the mutation which causes the change from local lesion-forming to systemic type is not known. When these mutant strains were taken from the very young systemically infected leaves and re-inoculated onto fresh White Burley plants, they again became systemic, without local lesion formation. Their original systemic occurrence was not therefore merely a result of a breakdown of the host localization reaction permitting the *flavum* type itself to go systemic.

There is reason to believe that the systemically spreading mutants had no effects on the development of lesions in the upper, challenge-inoculated leaves. Firstly, the systemic spread was initially into much younger leaves, and did not penetrate the challenged leaves until well after challenge inoculation. Secondly, when lesion development on challenge-inoculated leaves was followed in plants separated into groups with or without systemic symptoms, no differences in the effects of primary inoculation on lesion size or number in the challenge-inoculated leaves were found between the two groups.

To compare the results obtained with the differential host with those from an *N* gene host grown under the same conditions, the experiment shown in Fig. 1 was repeated with Burley S_3 tobacco. Table 1 summarizes the results. It is clear that formation of lesions on the primarily-inoculated leaves resulted in a reduction in size and number of lesions on the challenge-inoculated leaves, compared to the controls.

TABLE 1

Lesion numbers and diameters, and leaf areas, of challenge-inoculated leaves of Burley S_3 tobacco

Inoculum used for lower leaves[a]	TMV strain *vulgare*	Sterile phosphate buffer (control)	Per cent[c]
Lesions per 100 cm² of lower leaf	471	0	
Upper inoculated leaf:[b]			
Lesion diameter (mm)	2·31 ± 0·6	3·91 ± 0·8[d]	59
Leaf area (cm²)	539 ± 17	778 ± 45	69
Lesions per half leaf	23·8 ± 4·0	88·1 ± 7·4	27
Lesions per 100 cm² leaf area	8·7 ± 3·0	22·1 ± 7·4	39

[a] Lower leaves were inoculated with TMV strain *vulgare* at a dilution of $1 : 2 \times 10^2$, or with sterile phosphate buffer.

[b] Upper leaves of all plants were challenge-inoculated with TMV strain *vulgare* at a dilution of $1 : 10^3$.

[c] Values for plants inoculated with *vulgare* on the lower leaves, expressed as a percentage of the controls.

[d] For all parameters measured, the differences between the two treatments are statistically significant at least at the $P = 0{\cdot}05$ level. Values are means ± s.e.m.

Leaf area of the challenge-inoculated leaves also was reduced as a result of lesion formation on the lower leaves. The reduction in all three parameters was larger than the corresponding reduction observed with the differential host (Fig. 1).

Thus both the *N* gene host and the differential host show the effect described as "acquired systemic resistance", with reduction in lesion size and number.

Virus accumulation in challenge-inoculated leaves

The reduction in lesion size and number in challenge-inoculated upper leaves of plants which had formed lesions on the lower leaves raises the question of whether virus multiplication was also reduced. Figure 2 shows the patterns of accumulation of TMV strain *flavum* in challenge-inoculated leaves of White Burley tobacco. The amount of TMV RNA accumulation in a local lesion reaction is very small. To facilitate measurement, a very high concentration of inoculum was used to produce the maximum number of lesions possible without causing necrotic collapse of the leaf. Lesions first became visible 3 to 4 days after challenge-inoculation, and increased in diameter at an approximately linear rate for at least 10 days thereafter (R. J. Whenham, unpublished results). Net TMV RNA accumulation stopped when the lesions became visible, by day 4, and the concentration did not rise during the period up to day 15 when the lesions were growing in size. Indeed, there was some slight decrease in TMV RNA concentration between 4 and 15 days, probably due to some degradation of virus as tissue became necrotic. During the period up to 15 days, there was no difference in TMV RNA concentration between the controls and plants previously inoculated on the lower leaves.

Lesion numbers and sizes for this experiment are summarized in Table 2. From these data, we can calculate the reduction in TMV RNA concentration which might be expected from the "resistance" of the challenge-inoculated leaves, if it were to be assumed that TMV RNA concentration is proportional to lesion number and lesion area. For the experiment shown in Fig. 2, it was calculated that the concentration of

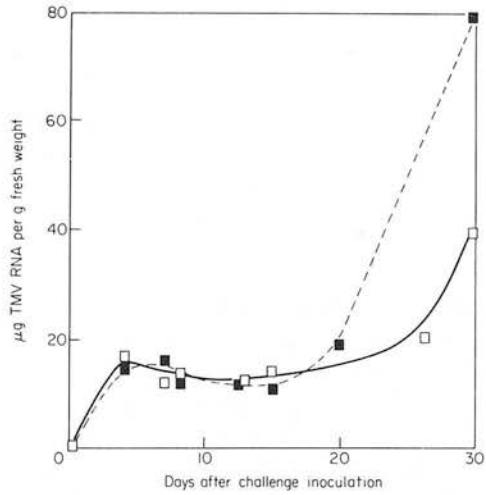

FIG. 2. Accumulation of TMV strain *flavum* RNA in challenge-inoculated leaves of White Burley tobacco. Upper leaves were challenge-inoculated with the local lesion-forming strain *flavum* at a dilution of $1 : 5 \times 10^2$. Ten days before the challenge-inoculation, the plants had been primarily inoculated with sterile phosphate buffer (controls) (□) or with TMV strain *flavum* at $1 : 10^2$ dilution (■). Each point is the mean of three determinations.

virus in the "resistant" leaves should be 39% of that in the control leaves (Table 2). Clearly no such difference existed (Fig. 2). Thus the reduced size and number of lesions in "resistant" leaves was not accompanied by a corresponding reduction in virus accumulation. The experiment shown in Fig. 2 and Table 2 was chosen as an extreme example, as the calculated expected reduction in TMV RNA concentration in the "resistant" leaves was large, and therefore would have been comparatively easy to detect. In replicate experiments, the calculated TMV RNA content of the "resistant" leaves ranged from 34 to 70% of the control level. Such differences in TMV RNA content should be detectable by the polyacrylamide gel assay method.

TABLE 2

Size and numbers of lesions on challenge-inoculated upper leaves of White Burley tobacco

Inoculum used for lower leaves[a]	TMV strain *flavum*	Sterile phosphate buffer (control)	Per cent[c]	
Lesions per half upper leaf[b]	468 ± 56	626 ± 20	75	$P < 0.05$
Mean lesion diameter (mm) at 10 days	2·78 ± 0·25	4·52 ± 0·30	61	$P < 0.01$
Mean lesion area (mm^2)	5·94	16·04	37	
Mean leaf weight (g)	4·53	6·30	72	
Calculated content of TMV RNA g fresh wt^{-1} (relative units)[d]	613	1593	39	

[a] Lower leaves were inoculated with TMV strain *flavum* at a dilution of $1 : 10^2$, or with sterile phosphate buffer.

[b] Upper leaves of all plants were challenge-inoculated with TMV strain *flavum* at a dilution of $1 : 5 \times 10^2$.

[c] Values for plants inoculated with *flavum* on the lower leaves, expressed as a percentage of the control values.

[d] Calculated assuming that TMV RNA per lesion is proportional to lesion area. Calculated TMV RNA/g = number of lesions × lesion area ÷ leaf weight.

The fact that no differences were detected between control and "resistant" leaves by this method substantiates the conclusions of Balazs *et al.* [2] based on infectivity assays.

After 15 days, the concentration of TMV RNA in the challenge-inoculated leaves began to rise again. This was because of the eventual invasion of the challenge-inoculated leaves by the naturally occurring systemic mutants. This systemic spread was earlier in those plants which had received a primary inoculation than in controls which received only the challenge inoculation (Fig. 2).

When the upper leaves of White Burley plants were challenge-inoculated with strain *vulgare*, a systemic infection resulted. TMV RNA accumulated to a very much higher concentration than when local lesions were formed. Figure 3 shows the patterns of accumulation in leaves of control plants, and in plants previously inoculated with *flavum* and forming lesions on the lower leaves. The two experiments shown used different concentrations of challenge-inoculum, which resulted in rapid and slightly delayed accumulation patterns. Again there was no evidence of any reduction in rate of viral RNA accumulation in the challenge-inoculated leaves of those plants which had been inoculated previously with *flavum* and had formed lesions on the lower leaves. Indeed, these plants seemed to accumulate TMV RNA faster than the controls for the first 10 days after inoculation, though later concentrations were very similar. Thus the "resistance" expressed as a reduction in lesion size and number does not reduce the multiplication of a systemic strain of the virus.

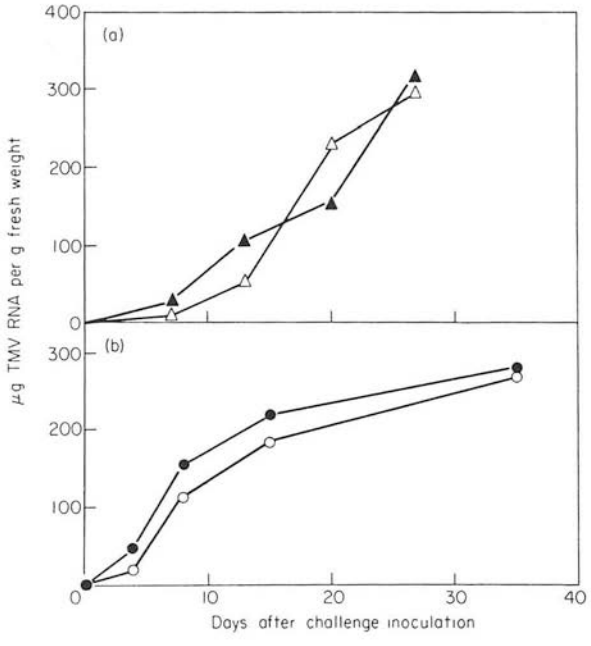

FIG. 3. Accumulation of TMV strain *vulgare* RNA in challenge-inoculated leaves of White Burley tobacco. Upper leaves were challenge-inoculated with the systemic TMV strain *vulgare* at dilutions of $1 : 5 \times 10^3$ (a) or $1 : 2 \times 10^2$ (b). Ten days before the challenge-inoculation, plants had been inoculated primarily on the lower leaves with sterile phosphate buffer (controls) (△, ○) or with the local lesion-forming strain *flavum* at a dilution of $1 : 10^2$ (▲, ●).

Systemic spread of the natural mutants arising from the primary *flavum* inoculation did occur in plants which were challenge-inoculated with *vulgare*. The systemic mutants could be distinguished from the normal systemic spread of *vulgare* by their extreme symptoms, which included yellow flecking and severe leaf crinkle. To avoid interference with the measurement of *vulgare* multiplication in challenge-inoculated leaves, any plants showing systemic mutant symptoms in the youngest leaves were discarded.

DISCUSSION

Is "acquired systemic resistance" actually resistance?

The results presented here show that formation of local lesions on the lower leaves of a differential host results in the development of fewer and smaller lesions when the upper leaves are inoculated subsequently with the same strain of virus. In these respects the results are similar to the "acquired systemic resistance" reported in varieties of tobacco containing the *N* gene for hypersensitivity [*6*, *12*, *20–22*]. The occurrence of "acquired systemic resistance" in a non-*N* gene tobacco variety shows that the phenomenon is not linked exclusively with *N* gene function, but can be induced by local lesion formation caused by other determinants. The occurrence of systemic resistance-type effects in other host–virus combinations [*21*] is further evidence that the effect is dependent generally on local lesion formation, and not on the activity of any single, specific genetic element.

The demonstration in White Burley that the accumulation of either systemic or local lesion strains of virus is not reduced in the challenge-inoculated leaves by previous lesion formation on lower leaves suggests that the phenomenon is not one of resistance to virus as such. Balazs *et al.* [*1*, *2*] reached a similar conclusion from results showing that infectivities of extracted virus in "resistant" and control challenge-inoculated leaves of Xanthi-nc tobacco were similar, although lesions on the "resistant" leaves were smaller and less numerous. In contrast, Ross [*21*] found that "resistant" leaves of Samsun NN tobacco had much lower relative infectivity than control leaves. The reason for the difference between these two reports is not clear. Van Loon & Dijkstra [*28*] reported that when Samsun tobacco (lacking the *N* gene) formed lesions on lower leaves in response to infection with tobacco necrosis virus, the amount of TMV accumulated in a subsequent systemic infection of the upper leaves was slightly reduced. This measurement was, however, made at only one time, shortly after challenge inoculation, and therefore cannot be compared with the results in Fig. 3.

A qualification must be attached to all these attempts to test for resistance by measuring TMV content, directly or by infectivity. Content depends on rate of virus synthesis and rate of loss by degradation during necrosis. A resistance mechanism might be expected to operate against TMV synthesis. Such resistance could operate without necessarily reducing TMV content: a slower rate of TMV synthesis could be balanced in "resistant" leaves by a slower rate of degradation during necrosis of the lesions. Measurements of lesion diamater (Fig. 1) shows that necrosis does proceed more slowly in "resistant" leaves. However, any such "cryptic" resistance clearly cannot operate against the systemic strain of virus, where no difference was detected

between final TMV RNA contents of control and "resistant" leaves (Fig. 3), and where loss of virus by necrosis does not occur. The possibility of resistance operating against TMV synthesis in necrotic lesions is being investigated further by direct measurement of synthetic rates.

Effects on lesion size and number

Ross [*21*] suggested that the acquired systemic resistance effects on lesion size and number were related: that reduction in numbers was a secondary effect stemming from such reduction in size that the lesion was no longer visible. However, there is reason to believe that the two effects may be produced by separate mechanisms, as they can vary independently in other situations. For example, in *N. glutinosa*, we found that lesion formation on the lower leaves resulted in slightly smaller lesions on challenge-inoculated upper leaves, but these lesions were up to 3·5 times more numerous than on challenged control leaves [*8*]. Cassells *et al.* [*7*] found a reduction in lesion number in Xanthi-nc tobacco, but no effects on lesion size.

The mechanism causing lesions on upper leaves of previously inoculated White Burley plants to be smaller than those of control plants need not be related to the mechanism responsible for preventing spread of virus from near the site of infection as part of the normal local lesion response. This restrictive mechanism [*15*] obviously operates in both cases. Indeed, the equal concentrations of TMV RNA accumulated in leaves showing large and small lesions (Fig. 2; Table 2) suggests that the mechanism restricting virus spread operates equally well in the two cases. Balazs *et al.* [*1, 2*] have suggested that the slower growth in size of necrotic lesions on "resistant" leaves may be caused by alterations in leaf cytokinin content as a result of lesion formation on lower leaves.

A likely explanation for altered lesion numbers on the challenge-inoculated leaf is that the number or susceptibility of infectible sites is altered if the lower leaves form necrotic lesions. Reduction in the number of infected sites might be expected to reduce the amount of virus synthesized if the amount of virus per lesion is the same for all treatments. However, the reduction in upper leaf size as a consequence of lesion formation on lower leaves tends at least partly, to cancel out the effects of reduction in lesion numbers, when TMV RNA is calculated per g leaf (Table 2). In *N. glutinosa*, where the number of lesions on the upper leaves can be increased by 3·5 fold as a result of previous inoculation of the lower leaves, without significant effect on upper leaf size, the TMV RNA concentration is also three times higher than that in control upper leaves [*8*].

Cassells *et al.* [*7*] have suggested that the reduced number of lesions produced in Xanthi-nc tobacco in which "systemic resistance" had been induced by polyacrylic acid might be caused by increased water stress in the challenged leaves: less turgid, more stressed leaves are known to be less easy to infect [*13, 16, 19*]. The converse situation of "acquired systemic susceptibility" in *N. glutinosa* also offers support for this hypothesis. We found that the increased lesion number on upper leaves of plants previously inoculated and forming lesions on the lower leaves was associated with a reduced endogenous concentration of abscisic acid in the challenged leaves [*8*]. This

suggests that these leaves were under less water stress than leaves on control plants [*5*, *31*], were more turgid and hence easier to infect [*4*, *24*, *30*].

In White Burley, we have found that the abscisic acid concentration of upper, non-infected leaves of plants inoculated with *flavum* on the lower leaves is about twice that in sham-inoculated control plants (R. J. Whenham & R. S. S. Fraser, in preparation). This difference persists for 15 to 25 days after the inoculation of the lower leaf. It suggests that the upper leaves may, as a result of lesion formation on the lower leaves, become less turgid and hence less easy to infect [*13*, *19*] than leaves on control plants.

All these sources of evidence therefore suggest that alteration in leaf turgidity as a result of inoculation of lower leaves is the most important factor causing altered lesion number on the challenged leaf. Alteration of lesion size is not covered by this mechanism, and still awaits a complete explanation. However, as shown above, lesion size can be reduced without concomitant reduction in virus accumulation. This suggests that reduction in lesion size is not necessarily evidence for a resistance mechanism, but may reflect an alteration in the ability of the host to become necrotic, without altering the mechanism restricting virus spread and accumulation.

I thank Su Loughlin for excellent technical assistance. Burley S_3 seed was from Centre Suisse de Researche sur la Tabac, Nyon, Switzerland.

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Printed in Great Britain by John Wright and Sons Ltd. at The Stonebridge Press, Bristol BS4 5NU

J. gen. Virol. (1980), **46**, 139–148
Printed in Great Britain

Tobacco Mosaic Virus Infection Does Not Alter the Polyadenylated Messenger RNA Content of Tobacco Leaves

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(*Accepted* 3 *August* 1979)

SUMMARY

Measurements of incorporation of ^{3}H-histidine into proteins in discs from tobacco mosaic virus-infected leaves suggest that the rate of host protein synthesis is reduced by up to 75% during virus multiplication but then recovers. Polyadenylated messenger RNAs from healthy and virus-infected plants were found to have similar size distributions and polyadenylic acid chains of similar lengths. Tobacco mosaic virus infection did not cause any alteration in the concentration of host polyadenylated messenger RNA. This makes it unlikely that rates of transcription or turnover of host polyadenylated messenger RNA were altered by infection. It is suggested that inhibition of host protein synthesis during virus multiplication may result from controls at the translational level, possibly by competition between the messenger RNAs for virus and host proteins.

INTRODUCTION

When tobacco leaves are infected with tobacco mosaic virus (TMV) the virus coat protein becomes the commonest single protein and may eventually form as much as three quarters of the total protein content. Early studies of protein metabolism in infected leaves suggested that coat protein was synthesized at the expense of host protein components (Wildman *et al.* 1949; Meneghini & Delwiche, 1951; Bawden & Kleczkowski, 1957) and that the rate of host protein synthesis was reduced after infection (Doke & Hirai, 1970; Hirai & Wildman, 1969).

The ways in which the virus may divert host protein synthesizing capacity to production of virus protein are not known. One possibility is that controls of transcription, translation or turnover of host messenger RNA (mRNA) might be altered. It was therefore of interest to examine host mRNA metabolism in TMV-infected leaves.

Many plant mRNAs contain 3′-polyadenylic acid sequence [poly(A); Higgins *et al.* 1973; Gray & Cashmore, 1976); in contrast, the mRNA for TMV coat protein is not polyadenylated (Siegel *et al.* 1973, 1976). This difference makes it possible to examine the metabolism of a defined fraction of host mRNA in healthy and infected tissues. We have measured the effects of TMV infection on leaf content of polyadenylated mRNA [poly(A)mRNA] by molecular hybridization between the poly(A) sequences and ^{3}H-polyuridylic acid.

METHODS

Plants and viruses. Tobacco plants (*Nicotiana tabacum* L. cv. Samsun) were grown in John Innes No. 2 compost in 12·5 cm diam. pots. The plants were kept in a glasshouse under natural lighting conditions, the temperature being 16 °C at night and between 20 and 25 °C during the day. TMV strains Rothamsted, *vulgare* (pale-green/dark-green mosaic) and

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flavum (severe yellow-green mosaic) (Melchers, 1940) were used. Inocula were prepared by grinding infected leaves with 1000 vol. of 50 mM-sodium phosphate, pH 7·0, using a pestle and mortar.

Leaves on plants 15 to 20 cm tall were dusted with 400-mesh Carborundum and inoculated by rubbing with virus suspension. Comparable leaves on control plants were sham-inoculated by rubbing with sterile phosphate buffer. The leaves were washed with tap water immediately after inoculation. Lengths of leaves inoculated in each experiment are given in Results.

Incorporation of ^{3}H-histidine into protein. Discs 5 mm in diam. were punched from the laminas of healthy and infected leaves. For each treatment, three replicate samples, each of four discs, were taken from three leaves. The discs were floated on water containing 1 μCi/ml L-2,5-^{3}H-histidine (sp. act. 43 Ci/mmol; Radiochemical Centre, Amersham, U.K.). The discs were infiltrated under vacuum for 2 min, then incubated for 1·5 h in a growth cabinet at 25 °C with an irradiance of 50 W/m^2. Control experiments showed that linear uptake and incorporation of histidine occurred from 20 min after the start of the radioactive incubation.

At the end of the radioactive incubation, the discs were rinsed with distilled water and lightly blotted. They were then heated to 90 °C for 15 min in 0·6 ml 0·5 N-perchloric acid, to hydrolyse any transfer RNA charged with radioactive histidine (Bretthauer *et al.* 1963). This procedure extracts RNA from discs without prior homogenization. After cooling on ice, the discs were homogenized and insoluble material was sedimented by centrifugation at 12000 *g* for 1 min. A sample of 0·3 ml of the supernatant was counted in 10 ml scintillation fluid [5 g/l 2-(4′-*t*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole (butyl-PBD) in 60% (v/v) toluene; 40% (v/v) 2-methoxyethanol], to measure acid soluble radioactivity. The precipitate was collected by filtration on a 21 mm diam. Whatman GF/C glass fibre disc, washed four times with 2 ml 7·5% (w/v) trichloroacetic acid and dried. Acid-insoluble radioactivity on the discs was measured by counting in 4 ml 0·5% (w/v) butyl-PBD in toluene.

Acid-soluble and acid-insoluble radioactivities were converted to disintegrations per min using known activities of 5-^{3}H-polyuridylic acid [poly(U)] and ^{3}H-toluene as insoluble and soluble internal standards. Acid-insoluble radioactivity was a measure of histidine incorporation into protein. Isotope uptake per disc was the sum of the acid-soluble and acid-insoluble radioactivities.

Nucleic acid extraction and TMV RNA measurement. Total nucleic acid was extracted from 0·5 to 1 g samples of leaf by a detergent–phenol procedure and purified by re-precipitations and dialysis as described elsewhere (Fraser & Whenham, 1978*a*, *b*). Total yield of nucleic acid was measured from the u.v. absorption spectrum of the extract. TMV RNA content of the extract was measured after polyacrylamide gel electrophoresis (Fraser & Whenham, 1978*a*). The amount of non-viral nucleic acid in a sample was calculated by subtracting TMV RNA content from the total nucleic acid content.

Measurement of poly(A) content. Fifty-microgram samples of total nucleic acid were washed as precipitates with 1 ml 0·1 M-NaCl in 80% (v/v) ethanol. The precipitate was dissolved in 1 ml 0·3 M-NaCl, 0·03 M-sodium citrate, pH 6 (2 × SSC), then mixed with 15 nCi ^{3}H-poly(U) (Miles Laboratories, Ltd, Slough, Bucks). Non-radioactive poly(U) (Sigma Chemical Co., Poole, Dorset) had been mixed with the radioactive poly(U) to give a total poly(U) content of 0·3 μg per sample.

The nucleic acid–poly(U) mixture was heated to 90 °C for 5 min, cooled to room temperature for 1 h, and 40 μg pancreatic ribonuclease (RNase) added. Non-hybridized poly(U) was digested at room temperature for 30 min. RNase-resistant poly(A).poly (U) hybrid was precipitated by addition of 500 μg bovine serum albumin carrier and 2 ml ice-cold 7% (w/v) trichloroacetic acid. After 15 min at 0 °C, the precipitate was collected by filtration on

a GF/C disc and washed three times with 2 ml 7% trichloroacetic acid. The disc was air-dried and ^{3}H radioactivity estimated by scintillation counting as above.

Control experiments showed that saturation of the poly(A) present in 50 μg total nucleic acid was obtained by hybridization with 0·1 μg poly(U). Use of 0·3 μg poly(U) per sample thus ensured an adequate excess. Under the conditions of hybridization used, the hybrid between poly(A) and poly(U) is not degraded by RNase (Bishop *et al.* 1974). Our control experiments showed that no significant amount of ^{3}H-poly(U) was rendered acid soluble by RNase digestion when hybridized under these conditions with an excess of non-radioactive poly(A) (Miles Laboratories Ltd). Further control experiments showed that when ^{3}H-poly (U) was mixed with total nucleic acid, RNase digestion of unhybridized poly(U) was complete by 30 min under the conditions used and no further radioactivity was rendered acid-soluble by increasing the treatment time or RNase concentration.

Sucrose density gradient fractionation of RNA. Between 200 and 500 μg total nucleic acid was dissolved in 0·5 ml 2 × SSC and fractionated by sedimentation through a 32 ml linear gradient of 5 to 25% sucrose in 2 × SSC. Centrifugation was for 18·5 h at 25000 rev/min (53000 *g* at average radius) in the SW 25·1 rotor of the Spinco Ultracentrifuge, at 0 °C. After centrifugation, absorbance at 260 nm was monitored by upward displacement of each gradient through a flow cell. Between 28 and 33 fractions were collected from each gradient. The poly(A) content of each fraction was measured by hybridization as above, except that 0·53 μg poly(U) (26·5 nCi) was used for each assay.

Measurement of poly(A) length. Sixty-milligram samples of total nucleic acid were prepared from plants infected with the *vulgare* strain of TMV and from comparable healthy plants. Poly(A)mRNA was separated from total RNA by affinity chromatography on a 100 mm long, 12 mm diam. column of poly(U)–Sepharose 4B, as described by Trapy & Esnault (1978), except that binding was at 0·4 M-NaCl and elution at 60 °C. Poly(A) was prepared from the poly(A)mRNA by digestion of non-poly(A) regions with RNases A and T_1 as described by Covey & Grierson (1976), except that poly(A) was ethanol-precipitated without addition of carrier.

The length of the poly(A) sequence was measured by electrophoresis in polyacrylamide gels in the presence of 99% formamide, to ensure denaturation and to eliminate base stacking (Staynov *et al.* 1972). The gels had final concentrations equivalent to 10% acrylamide and 0·42% *N,N'*-methylenebisacrylamide. Electrophoresis was for 12 h at 1·25 mA/gel constant current. Poly(A) preparations of known mean sequence lengths (Miles Laboratories Ltd.) were used as size standards.

After electrophoresis, the gels were washed for 5 h in distilled water to remove formamide and the positions of poly(A) peaks were determined by scanning at 265 nm in a Joyce–Loebl gel scanner. The approximate length of tobacco leaf poly(A) sequences was determined using the linear relationship between log mol. wt. and electrophoretic mobility (Staynov *et al.* 1972).

RESULTS

Inhibition of host protein synthesis during virus multiplication

In previous reports on the reduction of host protein content and synthesis in TMV-infected leaves (Wildman *et al.* 1949; Bawden & Kleczkowski, 1957; Hirai & Wildman, 1969; Doke & Hirai, 1970) the extent of any overall inhibition of the rate of host protein synthesis was not quantified. Before considering what mechanisms might underlie a reduction in rate of host protein synthesis, it was necessary to measure the extent and duration of inhibition after infection.

TMV coat protein does not contain the amino acid histidine (Tsugita *et al.* 1960; Hennig

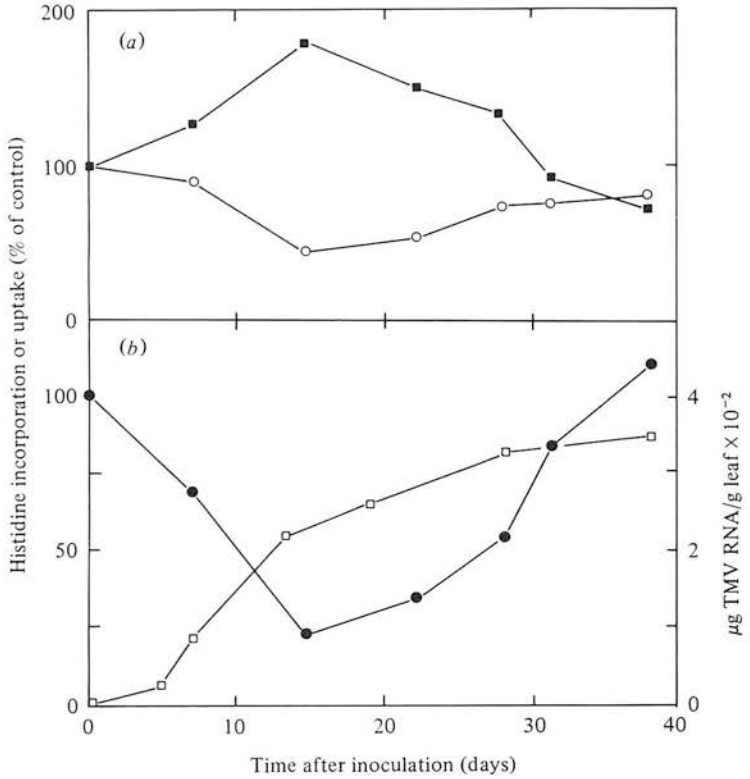

Fig. 1. Inhibition of host protein synthesis in tobacco leaves infected with tobacco mosaic virus. Leaves were inoculated with TMV strain *vulgare* just before reaching maximum length. (*a*) Changes in rates of histidine uptake (■—■) and incorporation (○—○) after infection, expressed as a percentage of the rates in comparable healthy leaves. (*b*) Changes in the rate of histidine incorporation per unit histidine uptake (●—●) in infected leaves, expressed as a percentage of the rate of histidine incorporation per unit histidine uptake in comparable healthy leaves; □—□, pattern of TMV multiplication, measured as TMV RNA accumulation.

& Wittman, 1972) and thus ^{3}H-histidine will only label host proteins and virus-specified proteins other than the coat protein (Sakai & Takebe, 1972). As the latter are synthesized in very small amounts (Sakai & Takebe, 1972), ^{3}H-histidine incorporation may be used for measurement of the rate of host protein synthesis in infected leaves.

Fig. 1. shows that during TMV multiplication, the rate of histidine incorporation in discs from infected leaves was reduced to about 50% of the rate in discs from comparable healthy leaves. However, the rate of histidine uptake by discs from infected leaves was greater than in discs from healthy leaves during TMV accumulation. The inhibition of histidine incorporation in infected leaves may therefore have been partly masked by the stimulation of uptake. In an attempt to minimize this effect, rates of histidine incorporation in infected and healthy leaf discs were divided by the histidine uptake values for these discs, to give data for rate of incorporation per unit uptake. Fig. 1 (*b*) shows the rate of incorporation per unit uptake in infected leaf discs as a percentage of the rate in healthy discs. The data show that during virus accumulation, the rate of histidine incorporation per unit uptake was reduced by 50 to 75%. It then recovered to the healthy leaf level after TMV accumulation had ended.

The data shown in Fig. 1 were obtained with leaves inoculated just before they reached maximum length. We have also studied leaves inoculated when at one-third of their final length, and leaves which became infected by systemic spread of virus when less than one-tenth of their final length. In these cases also, the rate of histidine incorporation in in-

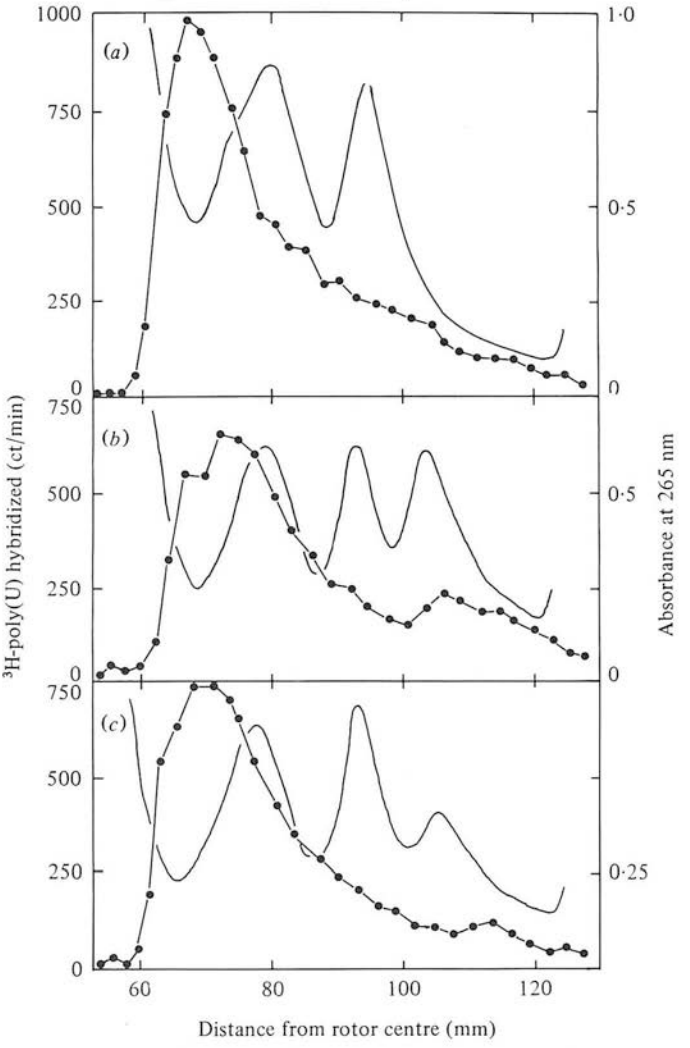

Fig. 2. Fractionation by sucrose density gradient centrifugation of nucleic acids from tobacco leaves. Sedimentation was from left to right: (*a*) healthy leaves; (*b*) infected with TMV strain Rothamsted; (*c*) infected with TMV strain *flavum*. Virus-infected leaves were harvested 13 days after inoculation, during the period of rapid virus multiplication. The continuous line shows absorbance at 260 nm. The peaks are: at 60 mm, transfer RNA plus low mol. wt. ultraviolet-absorbing contaminants; at 80 mm, 18S ribosomal RNA and DNA; at 90 to 100 mm, 25S ribosomal RNA, and at 100 to 110 mm, TMV RNA; ●—●, amount of ^{3}H-poly(U) hybridized to each gradient fraction.

fected leaf discs, adjusted for uptake stimulation, was reduced by 50 to 75% during virus accumulation, then recovered to the healthy leaf level after virus accumulation had ended. These data therefore suggest that the reduction in rate of host protein synthesis as a result of TMV infection is large, but that it only persists while TMV accumulation is occurring.

A reservation must be attached to quantification of rates of synthesis based on incorporation of radioactive precursors. Any changes in precursor pool size or turnover kinetics as a result of infection could cause changes in rate of incorporation not due to changes in the rate of synthesis. However, two independent lines of research support our estimate of the extent of inhibition of host protein synthesis. Firstly, the rate of net accumulation of host protein during growth of young leaves is reduced by 60 to 70% by TMV infection. Secondly, the rates of incorporation of ^{35}S-sulphate into the three commonest host proteins (chlorophyll *a*

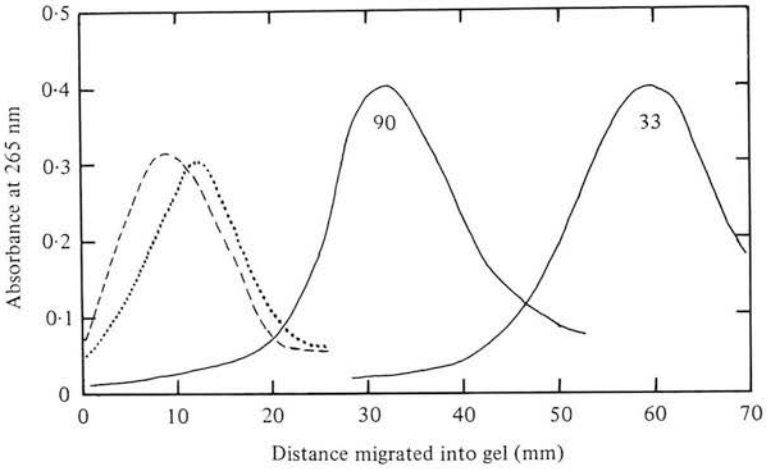

Fig. 3. Measurement of poly(A) size by electrophoresis on polyacrylamide gels in formamide. Peaks marked 90 and 33 are commercial poly(A) preparations with mean chain lengths 90 and 33 A residues. — — —, Poly(A) isolated from healthy leaves; ....., poly(A) from TMV strain *vulgare*-infected leaves.

binding protein, and the large and small subunits of ribulose 1,5-diphosphate carboxylase) are also reduced by around 75% during TMV multiplication (R. Fraser, J. Connor & A. Gerwitz, unpublished results).

As all these results indicate a severe inhibition of host protein synthesis during TMV multiplication, it was relevant to ask whether this was brought about by a corresponding reduction in host mRNA content, or by other means.

Hybridization with ^{3}H-poly(U) as a measure of poly(A)mRNA content

Molecular hybridization between poly(A) and the complementary homopolynucleotide, ^{3}H-poly(U) measures the total sequence length of poly(A) in a nucleic acid preparation. Two types of experiment were necessary to establish that poly(A) content is a valid measure of poly(A)mRNA content.

Fig. 2 shows fractionations by sucrose density gradient centrifugation of poly(A) RNAs from healthy and TMV-infected leaves. In each case, the poly(A) RNAs had a polydisperse distribution, from 6S to over 30S, with a peak at 9 to 13S. This distribution is consistent with the expected sizes of a natural population of messenger RNAs coding for a range of mol. wt. of proteins. There was no evidence of any partial degradation of poly(A)mRNA in virus-infected plants: this would have been detected as a shift in the distribution of the poly(A) RNAs to lighter regions of the gradient. In some nucleic acid preparations from infected plants we found a very small peak of hybridizing material at 2 to 3S, which probably represented a trace of free poly(A) unattached to mRNA.

Differences in poly(A) contents between samples of nucleic acid could arise not only if the samples contained different numbers of poly(A)mRNA molecules, but also if the length of the poly(A) sequence attached to each of these molecules was different in the two samples. The data in Fig. 3 indicate that the poly(A) sequences in nucleic acids extracted from healthy and TMV-infected leaves were of similar mean length and length distribution. The apparent size, based on extrapolation from the two markers of known sequence lengths, was 160 A residues for TMV-infected leaves and 180 for healthy leaves. These values are in good agreement with other reports of plant poly(A) sequence lengths (Sahger *et al.* 1974; Covey & Grierson, 1976).

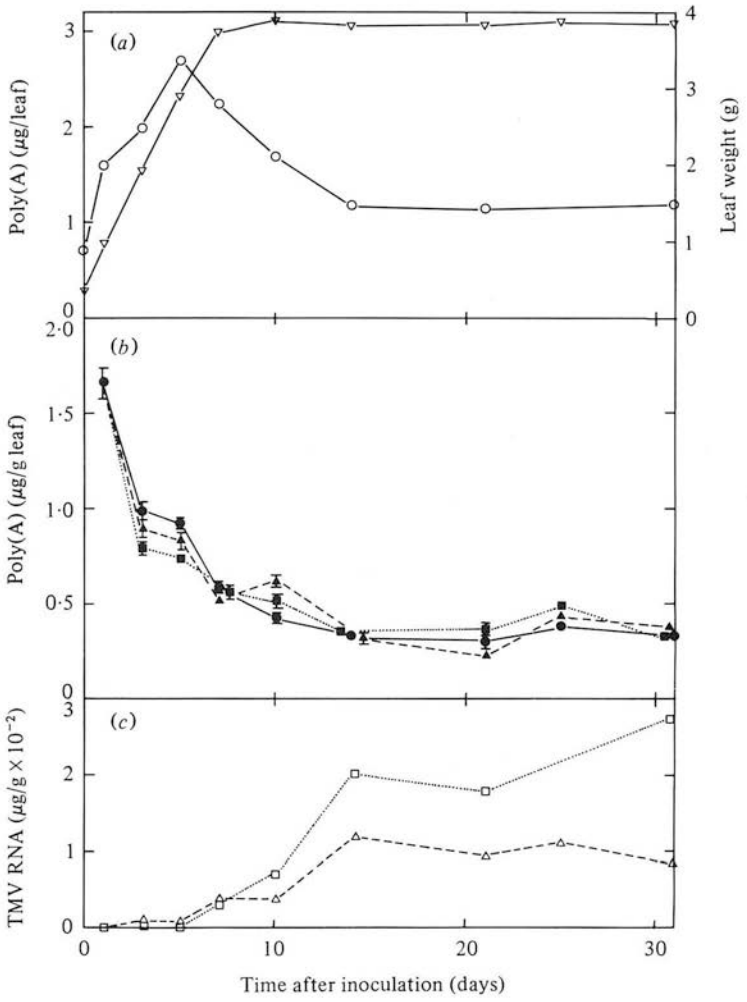

Fig. 4. Changes in poly(A) contents of healthy and TMV infected leaves. (*a*) Changes in poly(A) per leaf (○—○) and in leaf fresh weight with leaf age (▽—▽) for healthy leaves. (*b*) Changes in poly(A) per g fresh weight of leaf with time for healthy leaves (●—●) and for leaves inoculated with TMV strains Rothamsted (■···■) or *flavum* (▲--▲) at day 1. Each point is the mean of five or six determinations; the horizontal bars show standard errors of the means of replicate determinations of poly(A) content on 50 μg samples of nucleic acid from each bulk nucleic acid extract. Where no bars are shown, standard errors were smaller than the symbol size. (*c*) Patterns of multiplication of TMV strains Rothamsted (□····□) and *flavum* (△--△) in the infected leaves, measured as TMV RNA accumulation.

Having shown that the poly(A) sequences of healthy and infected leaves are of similar length, and are attached to RNA molecules of similar size distribution, we conclude that poly(A) content is a valid measure of the amount of polyadenylated RNA, presumed to be messenger RNA, in a nucleic acid sample. This conclusion holds for weight and for number of poly(A)mRNA molecules.

TMV infection and leaf content of poly(A)

Poly(A) content of healthy tobacco leaves rose to a peak just before the leaf reached its maximum weight, then declined as the leaf aged (Fig. 4*a*). A very similar pattern has been

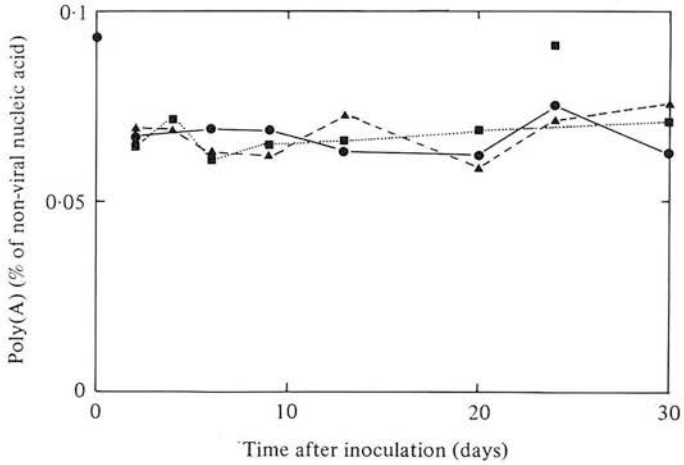

Fig. 5. Changes in poly(A) as a percentage of non-viral nucleic acid with age of leaf. Data were calculated from results shown in Fig. 4. ●—●, Healthy leaves; ■····■, leaves infected with TMV strain Rothamsted; ▲--▲, leaves infected with TMV strain *flavum*. Inoculation was at day 0. Each point is the mean of five or six hybridizations with ^{3}H-poly(U).

Table 1. *Poly(A) contents of healthy tobacco leaves, and of leaves infected with tobacco mosaic virus very early in development**

Days after inoculation	TMV strain	Leaf weight (g)	TMV RNA (μg/g leaf)	Poly(A) content (μg) per g leaf	Poly(A) content (μg) per 100 μg non-viral nucleic acid
9	—	0·29	—	2·56±0·09	0·115±0·004
	flavum	0·16	76	2·14±0·08	0·107±0·004
	vulgare	0·10	63	2·27±0·12	0·093±0·005
17	—	1·73	—	0·63±0·02	0·063±0·002
	flavum	1·23	123	0·81±0·05	0·107±0·007
	vulgare	1·14	372	0·63±0·04	0·103±0·006

* Tobacco plants were inoculated on expanded, lower leaves at day 0, and very young upper leaves became infected by systemic spread of virus. Control plants were sham-inoculated at day 0, and upper leaves comparable to those of virus infected plants harvested. Values for poly(A) contents are means ± standard errors of five replicate hybridizations carried out on 50 μg samples from each total nucleic acid extract.

found for amounts of ribosomal and transfer RNA per leaf (Fraser, 1972). Poly(A) per g fresh weight of healthy leaf fell during leaf development (Fig. 4*b*).

For the experiment shown in Fig. 4, leaves were inoculated with TMV strains Rothamsted or *flavum* as they approached maximum poly(A) content. The *flavum* strain caused much more severe visible symptoms, but as Fig. 4(*c*) shows, did not accumulate to such a high concentration in the plant as the Rothamsted strain. For both strains of virus, the period of most rapid TMV RNA accumulation occurred as maximum leaf weight was reached, and covered the period of maximum leaf poly(A) content and the start of the subsequent decline. Fig. 4(*b*) shows that neither strain had any effect on the poly(A) concentration of the leaf.

In functional terms, a significant relationship is the amount of mRNA compared to the remainder of the protein synthesizing system of the plant, because host and virus mRNAs have to share this, and possibly compete for it. One measure of host protein synthesizing capacity is non-viral nucleic acid, which, apart from a minor (about 20%) contribution from DNA, consists of ribosomal and transfer RNAs. Fig. 5 shows that when poly(A) content is

expressed per unit protein synthesizing system in this way it remained relatively constant with leaf age, and was not altered by virus infection.

We have also investigated the effects of TMV infection on poly(A) contents of leaves infected very early in development, when they still had to synthesize almost all of their eventual poly(A)mRNA content. As minute leaves are difficult to inoculate mechanically, they were infected by systemic spread of virus from inoculated lower leaves. Table 1 shows that there was considerable inhibition of growth by TMV infection. Poly(A) content per g leaf was not altered appreciably by either strain of virus. Poly(A) per unit non-viral nucleic acid was higher in these very young leaves than in older leaves (an effect also shown by the earliest sample in Fig. 5), but was unaltered by TMV 9 days after inoculation. At 17 days after inoculation, poly(A) per unit non-viral nucleic acid was actually higher in infected leaves than in healthy leaves. This probably occurred because of the very severe inhibition of ribosomal RNA accumulation which occurs when very young leaves are infected (Fraser, 1972, 1973).

DISCUSSION

We have shown that TMV infection does not alter the poly(A) content, a measure of poly(A)mRNA content, of tobacco leaves infected when very young or when approaching maturity. However, during the period of most active virus accumulation, the rate of host protein synthesis appears to be reduced by up to 75%. These findings have implications for the mechanisms controlling the rate of host protein synthesis in virus-infected plants. They make it unlikely that the inhibition of host protein synthesis is effected by controls operating at the levels of mRNA synthesis or turnover: such controls would tend to alter the concentration of poly(A)mRNA. In contrast to our results, fungal infection of plants has been shown to alter the rate of poly(A)mRNA synthesis (Yoshikawa *et al.* 1977) and to stimulate degradation of polyribosomal mRNA (Simpson *et al.* 1979)

The most likely interpretation of our data is that the observed inhibition of host protein synthesis is brought about by controls having their effect at the level of mRNA translation. One possibility is that the inhibition could be by purely competitive means. Infected plants contain the same amount of host poly(A)mRNA per unit protein synthesizing machinery (ribosomal plus transfer RNA) as healthy plants, but also contain the coat protein messenger RNA (Siegel *et al.* 1973). If some component of the protein synthesizing system, such as ribosomes, amino acids or energy, were limiting the overall rate of protein synthesis, the presence of large amounts of coat protein mRNA would tend to reduce translation of host mRNA by competition. The recovery in rate of histidine incorporation to the same level as in healthy leaves after the end of TMV accumulation (Fig. 1) is consistent with a competitive inhibition of host protein synthesis during virus multiplication.

However, it is also possible that translational regulation of host protein synthesis could involve more complex controls. For example, TMV coat protein mRNA might have an inherently higher translational efficiency than host mRNA. This type of situation has been reported in virus-infected mammalian cells (Oppermann & Koch, 1976). Alternatively, the translational efficiency of host messenger RNA might be reduced as a result of TMV infection.

Plants also appear to contain significant amounts of non-poly(A)mRNAs, and some of these could possibly represent the products of a separate population of genes (Gray & Cashmore, 1976; Ragg *et al.* 1977). It is possible that alterations in synthesis, turnover or translation of this class of mRNA could also contribute to the observed reduction in host protein synthesis after infection.

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(*Received* 11 *May* 1979)

J. gen. Virol. (1980), **48**, 87–96
Printed in Great Britain

Resistance to Tobacco Mosaic Virus in Tomato: Effects of the *Tm-1* Gene on Virus Multiplication

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(*Accepted* 11 *December* 1979)

SUMMARY

The gene *Tm-1* in tomato plants is dominant for suppression of mosaic symptoms caused by tobacco mosaic virus isolates designated as tomato strain 0. Virus multiplication (measured either by virus RNA content or by virus coat protein content) was inhibited in plants containing *Tm-1*. Inhibition was greater in hosts homozygous for *Tm-1* (90 to 95%) than in hosts heterozygous for *Tm-1* (65 to 75%). Thus, inhibition of tobacco mosaic virus multiplication is *Tm-1* gene dosage-dependent; suppression of visible symptoms is not.

Inhibition of TMV RNA accumulation occurred in both directly inoculated and systemically-infected leaves of *Tm-1*-containing hosts, and was consistent from experiment to experiment. *Tm-1* inhibited accumulation of RNA of different isolates of strain 0 to different extents: two isolates causing yellow mosaic symptoms on susceptible plants were especially strongly inhibited.

In *Tm-1* hosts, there was a delay between inoculation and the first detection of virus multiplication, measured either as increase in virus RNA content or recoverable infectivity. The delay was about 8 days in heterozygous *Tm-1* hosts and about 16 days in homozygous *Tm-1* hosts. The implications of these results for the mode of action of the *Tm-1* gene resistance are discussed.

INTRODUCTION

Tobacco mosaic virus (TMV) causes a serious disease of tomato, but good control has been achieved by use of host resistance. Several sources of resistance have been found (reviewed by Pelham, 1966; Walter, 1967; Alexander, 1971; Fletcher, 1973). Of these, the resistance genes designated *Tm-1*, *Tm-2* and *Tm-2*^{2} have been studied most. However, we do not yet know the biochemical mechanisms by which these genes prevent virus multiplication or symptom development.

The *Tm-1* gene suppresses the visible mosaic symptoms caused by TMV, but detectable virus multiplication occurs (Clayberg, 1960; Pécaut, 1962, 1964; Pelham, 1966, 1972). We have studied the effects of the *Tm-1* gene on the amount and pattern of TMV multiplication, as a first approach to understanding how the gene works.

METHODS

Plants and viruses. Near-isogenic lines of tomato (*Lycopersicon esculentum* Mill. cv. Craigella) were obtained from Dr T. J. Hall, Glasshouse Crops Research Institute, Littlehampton, Sussex, U.K. The susceptible line contained no genes for resistance to TMV. The two resistant lines had the *Tm-1* gene in either heterozygous or homozygous form (Table 1).

0022-1317/80/0000-3955 $02.00

Table 1. *Symptoms and changes in chlorophyll content resulting from infection of three tomato lines of different TMV-resistant genotypes with TMV strain* o*

Host line	Genotype	Symptoms produced by TMV infection	Chlorophyll content (mg/g fresh wt)	
			Healthy	TMV-infected
GCR 26	+/+	Systemic mosaic	4·02 ± 0·02	2·19 ± 0·11
J 484	*Tm-1*/+	None	3·74 ± 0·01	3·55 ± 0·08
GCR 237	*Tm-1*/*Tm-1*	None	3·53 ± 0·05	3·59 ± 0·01

* Plants were inoculated with TMV at a concentration of 2 μg/ml. Eighteen days later, symptoms were observed and chlorophyll concentration determined on four replicate samples of upper, systemically-infected leaves. Values are means ± standard errors.

The plants were grown in 12·5 cm diam. pots in Levington compost/sand (3:1) and transferred to larger pots as necessary. Plants were kept in a glasshouse at 16 °C at night; during the day the temperature rose to between 23 and 30 °C, depending on the season. Natural lighting was supplemented in the winter months with morning and evening illumination from mercury vapour lamps giving an irradiance of 50 W/m². Day length was a minimum of 14 h.

Isolates of TMV tomato strains o and 1 (Pelham, 1972) and a strain o isolate producing yellow symptoms, designated as strain oy, were obtained from Dr Hall, GCRI. The tomato strain *flavum* (Melchers, 1940) and the tobacco strain *vulgare* were originally from the Max Planck Institut für Biologie, Tübingen, West Germany. All TMV strains were multiplied in tobacco (*Nicotiana tabacum* L. cv. Samsun) and purified by the method of Mundry (1957).

Plants of uniform height were selected for each experiment; over all experiments the plants ranged from 10 to 30 cm tall at the time of inoculation. Between 3 and 7 replicate plants were used for each treatment or host genotype. Plants were inoculated on two upper, expanded leaves by dusting with 400-mesh Carborundum and rubbing by hand with a suspension of purified TMV at 2 to 50 μg/ml in 50 mM-sodium phosphate buffer, pH 7. Leaves were washed with running tap water immediately after inoculation.

Measurement of TMV RNA. At each sampling time, leaflets were taken from all leaves inoculated for each treatment. Samples were also taken from the 4th and 5th leaves above the inoculated leaves. Midribs were removed and the laminas chopped roughly and mixed. Three sub-samples of 0·5 or 1·0 g were then taken from each sample. Nucleic acids were extracted as described by Fraser & Whenham (1978) and fractionated by electrophoresis on 2·1% polyacrylamide gels (Loening, 1969) at 4 V/cm, 2·5 mA/gel, for 6 h. TMV RNA concentration per g fresh weight of leaf was calculated from the peak area of the TMV RNA on the u.v. absorption scan of each gel (Fraser, 1971).

Slab gel electrophoresis of proteins. Samples of 0·5 g leaf were homogenized in 5 ml, pH 8·6, buffer (40 mM-boric acid; 41 mM-tris; 4%, v/v, 2-mercaptoethanol; 10%, w/v, sucrose; 0·75%, w/v, sodium dodecyl sulphate) and heated in a boiling water bath for 3 min to extract and denature proteins. Debris was removed by centrifugation at 12000 *g* for 3 min and the protein extract stored at −20 °C. Samples of up to 100 μl extract were fractionated on slab polyacrylamide gels 160 mm wide and 1 mm thick, comprising a stacking gel (5% acrylamide), 10 mm long, and a separating gel, 150 mm long, which consisted of a linear gradient of 10 to 30% acrylamide. Gel preparation and the discontinuous buffer system used were as described by Neville (1971). After electrophoresis for 12 h at 10 mA constant current per gel, the gels were stained with Page Blue G90 (CI 42655) and destained as described by Laemmli (1970).

Chlorophyll content. Chlorophyll was extracted from sub-samples of 0·5 g leaf from

systemically-infected leaves using 80% acetone and was estimated spectrophotometrically (Vernon, 1960).

Infectivity bioassay. Leaflets were harvested from inoculated leaves at various times after inoculation. The midribs were removed and weighed samples were stored at −20 °C until samples from all harvest times were to hand. Each leaf sample was ground with 3 vol. of 50 mM-sodium phosphate buffer, pH 7, using a pestle and mortar. The extract was filtered through muslin and used to inoculate 12 half-leaves of the local lesion host *N. tabacum* L. cv. Xanthi-nc. The plants had been trimmed to four expanded leaves each, 1 day before inoculation. The different inocula were distributed at random through the test plants, but each inoculum was applied to the same number of lowest, second lowest, etc. leaves. Leaves were dusted with Carborundum before inoculation and washed with running tap water after inoculation. Lesions were counted 5 to 7 days after inoculation.

Measurement of TMV particle concentration. Virus was purified from samples of 50 to 200 g systemically-infected leaves by polyethylene glycol precipitations (Gooding & Hebert, 1967) and further purified by three cycles of differential centrifugation (Mundry, 1957). Virus concentration of the final solution was measured by u.v. absorption spectrophotometry, taking an absorbance at 260 nm of 1·0 to equal 0·37 mg TMV/ml.

RESULTS

Effects of the Tm-1 *gene on symptoms and TMV multiplication*

In confirmation of the results of Pelham (1972), we found that the *Tm-1* gene is dominant for suppression of visible mosaic symptoms caused by TMV (Table 1). In either the heterozygous or homozygous form, *Tm-1* completely prevented the loss of chlorophyll in systemically-infected leaves normally resulting from TMV infection (Table 1).

We measured the amounts of TMV produced in susceptible and resistant plants by estimating their contents of TMV RNA and coat protein. Ultraviolet absorption scans of gels of nucleic acids showed that, in susceptible plants, TMV RNA accumulated to form a very large peak and became the commonest nucleic acid species in the leaf. The *Tm-1*/+ host showed a much smaller TMV RNA peak, while even less TMV RNA accumulated in *Tm-1*/*Tm-1* plants. There was no evidence that TMV RNA in resistant hosts was suffering from any partial degradation, which would have shown as a skewing of the peak to the more mobile side.

Fig. 1 shows time courses of TMV RNA accumulation in susceptible and resistant plants. In the inoculated leaves of +/+ plants, TMV RNA concentration rose to a maximum at 20 days after inoculation, then declined. The decline was probably not due to degradation of TMV RNA, but to continued leaf growth diluting out the TMV RNA after accumulation had slowed or stopped: leaf fresh weight continued to increase after 20 days when maximum TMV RNA concentration was reached. The pattern of TMV RNA accumulation in systemically-infected leaves of the susceptible host was similar, and not detectably later than in inoculated leaves with the sampling frequency used.

In inoculated leaves of the resistant hosts, TMV RNA accumulation was much reduced and the extent of reduction was *Tm-1* gene dosage-dependent (Fig. 1). The heterozygous host showed a TMV RNA content 30% of that in the +/+ control at 20 days after inoculation; in the homozygous resistant host the TMV RNA concentration was only 5% of that in the control.

The patterns of accumulation of TMV RNA in inoculated and systemically-infected leaves of resistant plants were very similar, though inhibition of TMV RNA accumulation by *Tm-1*/+ or *Tm-1*/*Tm-1* tended to be a little less in systemically-infected leaves than in

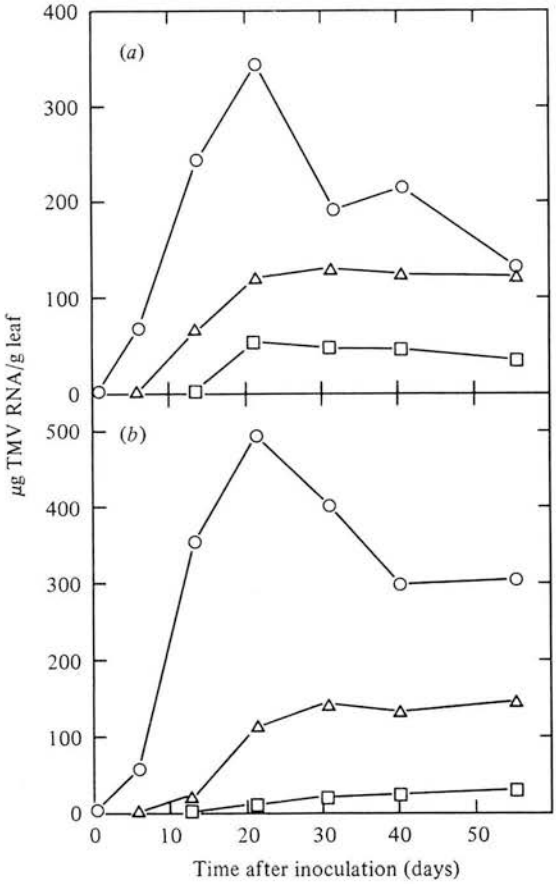

Fig. 1. Changes in TMV RNA concentration in TMV-infected tomato leaves. The host genotypes were: ○—○, +/+; △—△, *Tm-1*/+ and □—□, *Tm-1*/*Tm-1*. (*a*) Changes in leaves which became infected by systemic spread of virus; (*b*) changes in leaves directly inoculated with strain 0 at 10 μg/ml.

inoculated leaves. These results suggest that the resistance does not operate against systemic spread of the virus as such. In that case the multiplication of TMV in systemically-infected leaves of *Tm-1* plants would have been inhibited more than multiplication in inoculated leaves.

The inhibition of TMV RNA accumulation by *Tm-1* was long lasting: up to 56 days after inoculation, when the experiment ended, the TMV RNA concentration in the homozygous resistant host was still very much lower than in the control. Both resistant hosts showed a fairly steady concentration of TMV RNA from 20 to 56 days. As the leaves were visibly expanding during this time, it is probable that further virus synthesis was occurring, albeit at a low rate.

Fig. 2 shows that susceptible plants contained large amounts of coat protein. The intensity of the coat protein band was considerably less in the *Tm-1*/+ host, and coat protein was barely detectable in the homozygous resistant host. The effects of the *Tm-1* gene on accumulation of coat protein were thus similar to the effects on accumulation of TMV RNA.

In systemically-infected leaves of *Tm-1*/*Tm-1* plants which had a TMV RNA concentration 15% of that in comparable leaves of +/+ plants, the concentration of assembled TMV was also found to be 15% of the +/+ level. Therefore it is likely that the low amounts of

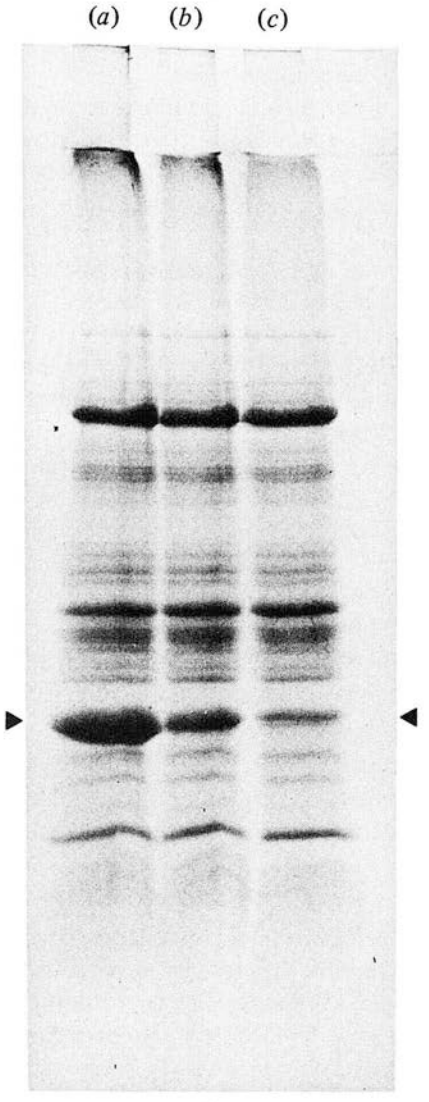

Fig. 2. Polyacrylamide slab gel electrophoresis of proteins from TMV-infected tomato leaves of genotypes (*a*) +/+; (*b*) *Tm-1*/+ and (*c*) *Tm-1*/*Tm-1*. The TMV coat protein band is indicated by arrows. Proteins were extracted from inoculated leaves 25 days after inoculation with TMV strain o at 10 μg/ml.

TMV RNA and coat protein present in resistant plants were assembled into intact virus particles.

Nature of the TMV accumulated in resistant hosts

In considering our results showing a small accumulation of TMV RNA in leaves of *Tm-1* hosts, it was necessary to ask whether this represented strain o virus which had achieved some multiplication despite the presence of *Tm-1* resistance, or whether it resulted from chance occurrence of strain 1 (Pelham, 1972). This arises either by mutation or by contamination of the initial strain o inoculum (Dawson, 1965, 1967; Pelham *et al.* 1970; Cirulli & Ciccarese, 1975; Dawson *et al.* 1979) and is able to overcome the *Tm-1* resistance.

No systemic mosaic symptoms were detected in any strain o-infected *Tm-1*/+ or *Tm-1*/

Tm-1 hosts, even in plants kept until 96 days after inoculation. There was therefore no sign of selection of strain 1 virus by this time, even though systemic spread of TMV was detectable in such hosts by 10 to 20 days after inoculation.

As a more stringent test, we also took symptomless, systemically-infected leaves from *Tm-1*/+ and *Tm-1*/*Tm-1* plants at times after inoculation with strain 0 when gel electrophoresis showed that they did contain TMV RNA. These leaves were homogenized in phosphate buffer and the diluted sap used to inoculate fresh +/+, *Tm-1*/+ and *Tm-1*/*Tm-1* plants.

Sap from all *Tm-1*-containing first hosts caused symptoms on all +/+ second hosts, confirming the presence of infectious TMV in the inocula. Inoculum from two out of eight *Tm-1*/+ first hosts, and from one out of 11 *Tm-1*/*Tm-1* first hosts caused mosaic symptoms on the *Tm-1*/+ and *Tm-1*/*Tm-1* second hosts, thus showing strain 1 characteristics. The symptoms produced by these inocula were visibly different from those produced by the GCRI isolate of strain 1 and from each other. It is thus likely that these variants originated by mutation or selection during multiplication of the strain 0 inoculum in either first or second host.

In the remaining cases (the overwhelming majority of plants tested), inoculum from first hosts containing the *Tm-1* gene caused no symptoms on second hosts containing this gene, even by 40 days after inoculation. Symptoms produced by purified virus of strain 0 on +/+ plants and by strain 1 on all three types of host, were well developed by 15 days after inoculation. These results therefore suggest that the virus multiplying in *Tm-1* plants continued to behave as strain 0. We were also unable to detect any increased ability of TMV to multiply in the second *Tm-1* host. We conclude that the limited amount of TMV multiplication observed in *Tm-1* plants was principally because *Tm-1* resistance permits a reduced amount of multiplication of strain 0 and was not significantly due to selection of TMV strains able to overcome the inhibition of multiplication.

The early part of TMV multiplication in Tm-1 hosts

A notable feature of both resistant hosts was that the first detection of TMV RNA after inoculation was much later than in susceptible hosts (Fig. 1). No TMV RNA could be detected in *Tm-1*/+ plants 6 days after inoculation, or in the *Tm-1*/*Tm-1* plants at 13 days. Thus during this 'eclipse' period, TMV RNA concentration remained below the limit of detection by this method, which is about 0·1 μg/g fresh weight.

To study the pattern of virus multiplication in the early part of infection of resistant hosts, we followed changes in infectivity. This method is just sensitive enough to detect TMV continuously after infection, provided that the tomato plants are inoculated with a high concentration of TMV. As a different batch of Xanthi-nc test plants was used to follow development of infectivity in each host, no inference can be drawn from differences in the absolute numbers of lesions produced by inoculum from different tomato hosts.

In +/+ hosts, there was a very rapid increase in infectivity after inoculation (Fig. 3). Both resistant hosts initially showed a decline in the level of recoverable infectivity, then an increase. The effect was clearest in the *Tm-1*/*Tm-1* plants, where infectivity declined for 15 days before beginning to increase.

The infectivity results are consistent with the assays of TMV RNA in showing a delay which is dependent on *Tm-1* gene dosage before the onset of detectable virus accumulation. Part of the infectivity recoverable in the early part of the experiment, before detectable increase in infectivity, may have been from virus remaining on the surface of the leaf and was unlikely ever to initiate infection. Such residual surface virus should not, however, interfere with the above interpretation of the timing of eventual increase in infectivity in resistant hosts.

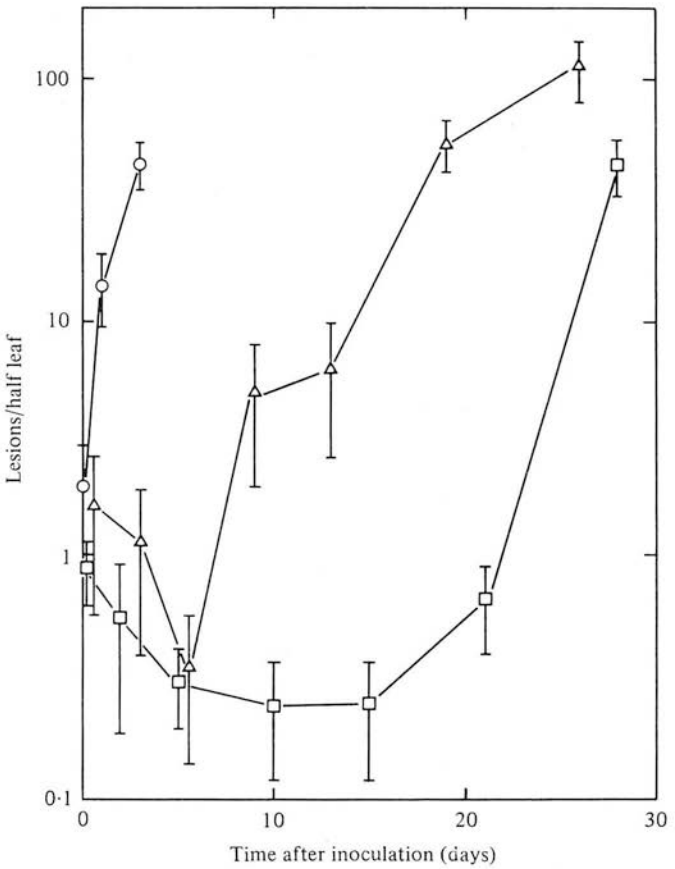

Fig. 3. Changes in infectivity recovered from tomato leaves with time after inoculation with TMV strain o at 50 μg/ml. The host genotypes were: ○—○, +/+; △—△, *Tm-1*/+ and □—□, *Tm-1*/*Tm-1*. Values are means ± standard errors.

Dawson (1965) found a delay between inoculation and measurable increase in infectivity in tomatoes of resistant line CStMW18. This line was originally reported to have three recessive genes for resistance to TMV (Walter, 1956), but has also been reported to contain *Tm-1* (Pécaut, 1964; Pelham, 1972).

Reproducibility of inhibition of TMV multiplication by Tm-1

Table 2 shows that in several experiments, the multiplication of TMV strain o was inhibited to similar extents by gene *Tm-1*. Inhibition was consistently more effective in homozygous than in heterozygous *Tm-1* hosts. The extent of inhibition was not affected by the time of year at which the experiment was done, nor by the size of plant at the time of inoculation.

To test whether the *Tm-1* inhibition of virus multiplication was equally effective against other isolates of strain o, plants were inoculated with three other isolates. Table 3 shows that none of these isolates multiplied quite as well in directly inoculated leaves of +/+ tomato as did the GCRI isolate of strain o. However, only strain oy multiplied less well than strain o in systemically-infected leaves.

Tm-1 resistance was effective against all strains, but not to the same extent. Multiplication of the two strains producing yellow-green mosaic symptoms (oy and *flavum*) was especially

Table 2. *Effects of the* $Tm\text{-}1$ *gene on multiplication of TMV strain* o *in inoculated and systemically-infected leaves of tomato hosts of different TMV-resistance genotypes*

Month	Height of plants at time of inoculation (cm)	Sampling time (days after inoculation)	TMV RNA content of resistant hosts*			
			Tm-1/+		*Tm-1/Tm-1*	
			Inoculated leaves	Systemic leaves	Inoculated leaves	Systemic leaves
July	30	28	18	26	—	—
	—	49	12	—	—	—
Sept.	15	21	25	34	2·2	15
Nov.	15	18	29	32	6·1	15
April	10	19	31	—	3·8	—
	—	28	—	32	—	1·4

* Expressed as percentage of TMV RNA content of comparable leaves on the +/+ susceptible host.

Table 3. *Concentrations of four type* o *isolates of TMV in inoculated and systemically-infected leaves of susceptible and resistant tomato plants**

TMV isolate	Host genotype			
	+/+		*Tm-1/+*	
	Inoculated leaves	Systemic leaves	Inoculated leaves	Systemic leaves
o	222	166	41 (18%)	43 (26%)
oy	57	119	2·5 (4%)	0 (0%)
flavum	59	169	1·5 (2·5%)	5·1 (3%)
vulgare	118	166	7·3 (6·2%)	43 (26%)

* Values are μg TMV RNA/g fresh weight, measured 28 days after inoculation with each strain of virus at 10 μg/ml. Figures in parentheses are TMV RNA concentrations in the *Tm-1/+* hosts expressed as a percentage of the concentration in comparable leaves of the +/+ host.

severely inhibited in *Tm-1* plants. Strain *vulgare* multiplication was inhibited to about the same extent as strain o.

DISCUSSION

Previous studies, using infectivity bioassay of TMV concentration, have suggested that the *Tm-1* gene either inhibits TMV multiplication (Pelham, 1972; Arroya & Selman, 1977; Motoyoshi & Oshima, 1977), has no effect on it (Clayberg, 1960; Pelham, 1972) or even stimulates it (Pelham, 1972). None of these reports gave a complete picture of virus multiplication in resistant plants. In our investigation, we chose not to use infectivity bioassay for two reasons. Firstly, unless very large numbers of test plants are used, infectivity bioassay is unable to distinguish between samples having small but possibly important differences in virus concentration (Bawden, 1950). Secondly, we have found that the specific infectivity of TMV multiplied in +/+ hosts is 5 to 15 times that of TMV multiplied in *Tm-1/Tm-1* hosts (R. S. S. Fraser & S. A. R. Loughlin, unpublished data). Thus comparison of TMV concentrations in different hosts by infectivity is likely to be unreliable.

Our results, based on direct measurements of TMV RNA and coat protein concentrations, show that the *Tm-1* gene consistently inhibited accumulation of both components of the virus. The differences in virus RNA content between +/+ and *Tm-1/+* plants, and between *Tm-1/+* and *Tm-1/Tm-1* plants, were of a size which would be difficult to detect by infectivity bioassay. But as TMV quickly came to form the predominant nucleic acid and

protein components of susceptible plants, its synthesis must have utilized a large proportion of host anabolic capacity. Reduction of TMV accumulation to 30% (*Tm-1*/+) or 10% (*Tm-1*/*Tm-1*) of the amount in +/+ plants would significantly reduce this drain on host synthetic capacity, and thus have less effect on growth.

From our results, certain suggestions can be made about the mode of action of the *Tm-1* gene. Measurements of virus concentration in resistant plants suggested that the limited amounts of TMV RNA and coat protein present were assembled into intact particles. This shows only that assembly can occur in *Tm-1* plants; it does not exclude the possibility that *Tm-1* resistance might act on the assembly process, with non-assembled TMV RNA and coat protein being degraded.

The greater inhibition of TMV accumulation in *Tm-1*/*Tm-1* plants than in *Tm-1*/+ plants suggests that the extent of inhibition is dependent on the concentration of gene product within the plant. In plants homozygous for *Tm-1*, both gene copies are presumably active, leading to a higher concentration of gene product than in the heterozygote with only one copy.

The pleiotropic effects of the gene on symptom suppression and inhibition of TMV multiplication give rise to an apparent paradox: the gene is dominant for symptom suppression, but inhibition of TMV multiplication is gene-dosage dependent. Various explanations are possible. A single active product of the gene might have the ability to suppress symptom formation at lower concentrations than those effective against virus multiplication; or the pathway between gene and active products could be branched, with separate products inhibiting symptom formation and multiplication. The third possibility is that the amount of virus accumulated in *Tm-1*/+ plants might be below some threshold concentration required for production of mosaic symptoms. However, if plants are grown at a constant 33 °C, TMV multiplies as well in *Tm-1* hosts as in +/+ hosts, but visible symptoms are still suppressed by *Tm-1* (our unpublished results). This suggests that the two effects of the *Tm-1* gene are due to partially separable mechanisms and that symptom formation is not solely controlled by amount of virus accumulated.

From the time courses of development of infectivity and accumulation of TMV RNA after inoculation of resistant hosts, it appears that one action of the antiviral gene is to delay the onset of detectable multiplication. The decline in infectivity recoverable from *Tm-1*/*Tm-1* plants from inoculation until 15 days later suggests that the resistance agent is not produced in response to an initial phase of virus multiplication, but is likely to be present in the plant before inoculation. In this feature Tm_1 differs from the *N* gene for TMV resistance in tobacco (Holmes, 1938), where an initial phase of virus multiplication occurs before the resistance mechanism becomes effective (Taniguchi, 1963; Fraser, 1979).

The patterns of TMV RNA accumulation and infectivity changes after inoculation of *Tm-1*/*Tm-1* plants further suggest that the stage of virus multiplication which is inhibited is an early one in the establishment of infection in the plant. If the susceptible stage were a comparatively late one, such as cell-to-cell spread of virus, then some initial rise in infectivity would be expected.

Having characterized some of the effects of *Tm-1* on TMV multiplication and symptom suppression, we are now in a position to ask more specific questions about which stage of virus multiplication is the target of resistance, and about the nature of the resistance gene product.

We thank Dr T. J. Hall, Glasshouse Crops Research Institute, for useful discussions and for supplies of tomato seeds and virus strains. We thank Jane Connor and David Brown for capable technical assistance.

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(Received 20 September 1979)

J. gen. Virol. (1980), 50, 221–224
Printed in Great Britain

Resistance to Tobacco Mosaic Virus in Tomato: Effects of the *Tm-1* Gene on Symptom Formation and Multiplication of Virus Strain 1

(*Accepted* 1 *April* 1980)

SUMMARY

The *Tm-1* gene in tomato inhibits development of mosaic symptoms and multiplication of tobacco mosaic virus (strain 0 isolates). A virus isolate of strain 1 type caused mosaic symptoms on *Tm-1* hosts almost as severe as those it caused on susceptible hosts. However, multiplication of strain 1 virus (measured as accumulation of virus RNA or coat protein) was still partly inhibited in *Tm-1* hosts. Thus the two end effects of the *Tm-1* gene were to some extent separable.

In tomato, the resistance gene *Tm-1* prevents the systemic mosaic symptoms caused by common (strain 0) isolates of tobacco mosaic virus (TMV; Clayberg, 1960; Pelham, 1972). Accumulation of TMV strain 0 is inhibited by about 70% in plants heterozygous for *Tm-1* and by 90 to 95% in plants with homozygous *Tm-1* (Fraser & Loughlin, 1980). Variants of TMV which can cause mosaic on *Tm-1* hosts have arisen naturally (Dawson, 1965, 1967; Pelham *et al.* 1970) and are designated as strain 1 (Pelham, 1972). As one approach to understanding how the *Tm-1* gene can suppress symptom formation and TMV multiplication, we have studied its effects on multiplication of TMV strain 1.

Nearly isogenic lines of tomato (*Lycopersicon esculentum* Mill. cv. Craigella) with different combinations of the *Tm-1* gene were grown under normal glasshouse conditions as described by Fraser & Loughlin (1980). Line GCR 26 is susceptible (+/+), J 484 is heterozygous (*Tm-1*/+) and GCR 237 is homozygous (*Tm-1*/*Tm-1*). TMV tomato strains 0 and 1, isolated by single lesion transfers on *Nicotiana tabacum* L. cv. Xanthi-nc, were multiplied in *N. tabacum* cv. Samsun and partially purified by the method of Mundry (1957).

Plants 15 to 30 cm tall were inoculated on two upper, expanded leaves by dusting with 400-mesh Carborundum and rubbing by hand with a suspension of TMV at 5 μg/ml in 50 mM-sodium phosphate buffer, pH 7. Between three and seven replicate plants were inoculated for each genotype. Leaves were washed with running tap water immediately after inoculation.

To eliminate subjective bias in assessing symptom severity, plants were scored 'blind', i.e. without the observers knowing host genotype or virus strain. Symptom severity was expressed on the scale: 0, symptom free; 1, trace of systemic mosaic; 2, well-developed systemic mosaic with light green/dark green mottling of young leaves; 3, severe mosaic, with pronounced mottling, stunting and distortion of the upper parts of the plant.

For measurements of TMV RNA and coat protein concentrations, leaflets were harvested from all inoculated leaves of each host type. Samples were also taken from the systemically-infected 4th and 5th leaves above the inoculated leaves. Midribs were removed and the laminas roughly chopped and mixed.

Nucleic acids were extracted from three 0·5 g samples of chopped lamina as described by Fraser & Whenham (1978). TMV RNA content was measured after fractionation by

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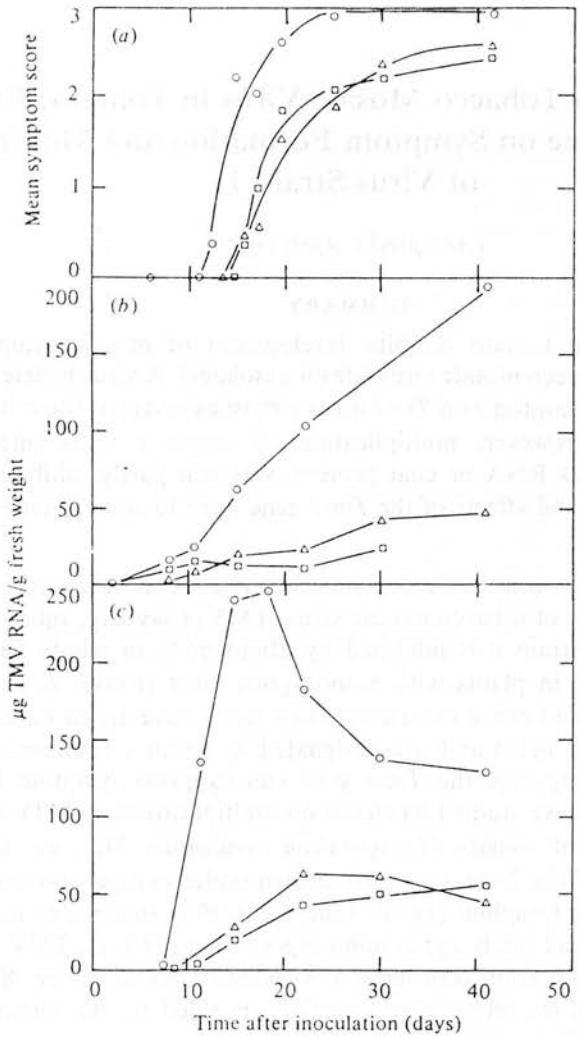

Fig. 1. Time courses of development of mosaic symptoms and accumulation of TMV RNA in susceptible and TMV-resistant tomato plants. Plants with genotypes +/+ (○—○); *Tm*-1/+ (△—△) and *Tm*-1/*Tm*-1 (□—□) were inoculated with TMV strain 1. (*a*) Development of systemic mosaic symptoms. (*b*) TMV RNA accumulation in inoculated leaves. (*c*) TMV RNA accumulation in systemically-infected leaves. Each point in (*b*) and (*c*) is the mean of three replicate determinations.

polyacrylamide gel electrophoresis (Loening, 1969) as described by Fraser & Loughlin (1980).

Total protein was extracted from 0·5 g samples and fractionated by electrophoresis under denaturing conditions on 10 to 30 % gradient polyacrylamide slab gels (Neville, 1971), as described by Fraser & Loughlin (1980). The gels were stained with PAGE Blue G90 and destained (Laemmli, 1970). After drying, the stained band corresponding to TMV coat protein (identified by co-electrophoresis with purified coat protein) was cut out and the stain eluted overnight into 4 ml formamide. Stain concentration in the eluate was measured by absorbance at 600 nm. The amount of stain bound and subsequently eluted was proportional to weight of coat protein over the range of sample sizes used. Each electrophoresis run was calibrated by fractionation of known weights of purified coat protein.

Table 1. *Concentrations of TMV RNA and coat protein in leaves of tomatoes of different resistance genotypes infected with TMV strain 0 or 1*

		TMV RNA (μg/g fresh weight)		TMV coat protein (mg/g fresh weight)	
Experiment*	Host genotype	Strain 0	Strain 1	Strain 0	Strain 1
1	+/+	221	258	—	—
	Tm-1/+	68	71	—	—
2	+/+	278	248	6·3	6·7
	Tm-1/+	56	111	2·0	3·5
	Tm-1/*Tm-1*	5	81	—†	2·2
3	+/+	364	318		
	Tm-1/+	115	120		
	Tm-1/*Tm-1*	5	79		

* Data for experiments 1 and 2 are from directly inoculated leaves; data for experiment 3 are from systemically-infected leaves. Sampling was at 28 days after inoculation for experiment 1, and at 19 days after inoculation for experiments 2 and 3. All values are means of three determinations.

† Coat protein concentration was below the limit of sensitivity of the method, i.e. was less than about 0·25 mg/g.

Fig. 1(*a*) shows that in susceptible hosts inoculated with strain 1, systemic mosaic symptoms became visible about 12 days after inoculation and quickly increased in severity. Susceptible plants inoculated with strain 0 developed symptoms at a similar rate (not shown). When inoculated with strain 1, mosaic symptoms did develop on *Tm-1*/+ and *Tm-1*/*Tm-1* plants, though more slowly and to a slightly lower final severity than on +/+ hosts. Plants with the *Tm-1* gene in homozygous or heterozygous form showed no mosaic symptoms when inoculated with strain 0 (Fraser & Loughlin, 1980).

Fig. 1(*b*) and (*c*) show time courses of accumulation of strain 1 TMV RNA in hosts of the three genotypes, in both inoculated and systemically-infected leaves. In the +/+ host, the fall in TMV RNA concentration in systemically-infected leaves after 20 days was probably not an indication of virus instability. Leaf expansion continued after maximum TMV RNA concentration had been reached, and may have diluted the virus after its rate of accumulation had slowed. We found similar patterns of accumulation of TMV RNA of strain 0 in the +/+ host (Fraser & Loughlin, 1980).

In leaves of hosts containing the *Tm-1* gene, accumulation of strain 1 TMV RNA was inhibited. The inhibition occurred both in inoculated and systemically-infected leaves. Twenty days after inoculation, TMV RNA concentration in *Tm-1* hosts was generally between 10 and 30% of that in susceptible hosts. Accumulation of the coat protein of strain 1 was also inhibited in *Tm-1* hosts, to about the same extent as TMV RNA accumulation (Table 1).

Further experiments compared the accumulation of strain 1 with that of strain 0 in susceptible and resistant plants. In susceptible plants, the two strains reached very similar concentrations, measured either as TMV RNA or coat protein (Table 1). In *Tm-1*/+ plants, inhibition of strain 1 accumulation was as great as or only slightly less than inhibition of strain 0 accumulation. In contrast, in *Tm-1*/*Tm-1* plants, strain 1 accumulation was consistently less inhibited than that of strain 0. The results show that, while inhibition of strain 0 accumulation was strongly dependent on *Tm-1* gene dosage, inhibition of strain 1 accumulation was not.

In *Tm-1*/*Tm-1* plants inoculated with strain 0, we found a delay of 20 days between inoculation and the first detectable increase in virus concentration (Fraser & Loughlin, 1980). In contrast, *Tm-1*/*Tm-1* plants inoculated with strain 1 did not show this 'eclipse' phase: detectable increase in TMV RNA was established by 6 days after inoculation (Fig. 1*b*).

Thus there were some differences in the effects of *Tm-1* on accumulation of strains 0 and 1, but strain 1 remained substantially unable to overcome the inhibitory effects of *Tm-1* on virus multiplication. Previously, we reported that accumulations of four different isolates of strain 0 were inhibited to different extents in *Tm-1* plants (Fraser & Loughlin, 1980). It is possible that accumulation of different isolates of strain 1 might also be inhibited to different extents.

The failure of strain 1 to overcome the inhibitory effect of *Tm-1* on virus accumulation contrasts with its ability to overcome the symptom suppression function of *Tm-1*. These results therefore suggest that the two end effects of *Tm-1* are to some extent separable and may involve partially independent mechanisms. One possible explanation for the dual function of the gene is that the pathway between gene and end effects is branched, with separate functional end products inhibiting symptom formation and virus multiplication. Alternatively, we cannot exclude the possibility that the sole effect of *Tm-1* is suppression of symptom formation, while inhibition of TMV accumulation is controlled by a separate gene only present in *Tm-1* plants.

We thank Dr T. J. Hall, Glasshouse Crops Research Institute, Littlehampton, U.K., for useful discussions and for supplies of tomato seeds and virus isolates.

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(*Received* 12 *February* 1980)

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Planta 150, 349–353 (1980)

Planta

Stimulation by Abscisic Acid of RNA Synthesis in Discs from Healthy and Tobacco Mosaic Virus-infected Tobacco Leaves

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Abstract. Uptake of abscisic acid from the culture medium by discs of healthy and tobacco mosaic virus-infected tobacco leaves was measured. Small (two to five-fold) increases in abscisic acid concentration in discs caused increases in rates of [^{3}H]uridine and [^{3}H]adenine incorporation into total nucleic acid, virus RNA and host ribosomal RNA. Net accumulation of virus RNA was also enhanced by abscisic acid. This evidence for stimulation of RNA synthesis is compared with previous reports showing inhibition of RNA synthesis in other tissues. It is suggested that the increase in endogenous abscisic acid caused by tobacco mosaic virus infection may be at least partly responsible for observed increases in rates of RNA synthesis after infection.

Key words: Abscisic acid – *Nicotiana* – Ribonucleic acid synthesis – Tobacco mosaic virus.

Introduction

When abscisic acid (ABA) is applied to plant tissues, its reported effects include inhibition of growth (reviewed by Milborrow 1974) and RNA synthesis (Villiers 1968; Pilet 1970; Bex 1972a; Zwar and Jacobsen 1972; Walbot et al. 1975; Minocha and DiBona 1979). The mechanism of inhibition of RNA synthesis is not understood: it has been suggested that ABA may indirectly interfere with chromatin activity (Pearson and Wareing 1969) and RNA polymerase activity (Bex 1972b). Infection of tobacco leaves by tobacco mosaic virus (TMV) increases their endogenous ABA concentration by two to five fold (R.J. Whenham and R.S.S. Fraser, submitted manuscript). RNA synthesis in virus-infected leaves is at a high rate: TMV RNA accumulates to as much as 500 µg per g fresh weight, and host ribosomal RNA synthesis is stimulated in the early stages of infection (Fraser 1972, 1973). These findings prompted us to examine the effects of increased ABA concentration on RNA synthesis in tobacco leaves. Our results suggest that in this tissue, small increases in ABA concentration may stimulate RNA synthesis and accumulation.

Abbreviations: ABA = abscisic acid; TMV = tobacco mosaic virus

Materials and Methods

Tobacco plants (*Nicotiana tabacum* L. cvs Samsun and White Burley) were grown and inoculated on two expanded lower leaves with TMV strain vulgare as described by Fraser and Whenham (1978). Upper leaves became infected by systemic spread of virus. Those which had expanded to about one-half final size were harvested one day after appearance of mosaic symptoms, i.e. very early in the virus multiplication phase (Fraser 1972). Comparable leaves were also taken from healthy plants.

After surface sterilization for 15 min in 0.01% benzalkonium chloride; 5% ethanol, leaves were washed three times with sterile water and discs of 6 or 15 mm diameter removed by cork borer. Groups of four discs were floated on sterile B5 medium (Gamborg et al. 1968) containing various concentrations of (±) *cis-trans*-abscisic acid (Sigma Chemical Co. UK) but no other hormones. Discs were infiltrated under vacuum for 2 min then incubated in a growth cabinet at 25° C with an irradiance of 50 W m^{-2} and a 14 h daylength. Each treatment was replicated three or five times.

[5-^{3}H]uridine (specific activity 0.9 TBq mmol^{-1}) or [2-^{3}H]adenine (specific activity 0.75 TBq mmol^{-1}) (Radiochemical Centre, Amersham USA) were added to label nucleic acids via the pyrimidine or purine precursor pathways respectively. Radioactive concentrations and time of labelling are given in results.

For measurement of total uptake and incorporation of radioactivity, each group of four discs was rinsed in water and lightly blotted, then homogenized in 1 ml 0.5 M $HClO_4$ at 0° C. The homogenate was centrifuged at 12,000 *g* for 3 min, and acid-soluble radioactivity measured by counting 0.1 ml of supernatant in 8 ml scintillation liquid (0.5% 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole in toluene: 2-methoxyethanol 6:4 by volume). Correction of counts to 100% counting efficiency was by the external standard channels ratio method.

The precipitate was suspended in 1 ml 0.5 M $HClO_4$, collected by filtration on a Whatman GF/A glass fibre disc and washed

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three times with 4 ml 0.5 M $HClO_4$. Nucleic acids on the filter were hydrolyzed at 70° C for 30 min with 0.3 ml 0.5 M $HClO_4$, then cold acid-insoluble radioactivity (incorporation) was determined by counting as above. Total isotope uptake was acid-soluble plus acid-insoluble radioactivity.

Amounts and rates of synthesis of individual RNA species were measured by extracting total nucleic acids by a phenol-detergent procedure as described by Fraser and Whenham (1978). The nucleic acids were fractionated by electrophoresis on 2.1% polyacrylamide gels (Loening 1969) for 3 h at 5 mA/gel, 8 V cm^{-1}. Gels were scanned for absorption at 265 nm and the amount of TMV RNA determined from the area of its ultraviolet absorption peak (Fraser 1971). Gels were cut into 1 mm thick slices and radioactivity in each slice measured by $HClO_4$ hydrolysis and scintillation counting as above.

ABA concentrations in healthy and infected leaf tissues were measured by extraction and gas-liquid chromatography as described by Fraser et al. (1979). ABA uptake by discs was measured by floating them on B5 medium containing various concentrations of total ABA and known radioactivities of (±) *cis-trans*-[2-^{14}C]abscisic acid (Radiochemical Centre, Amersham; specific activity 440 MBq $mmol^{-1}$). After incubation, the discs were rinsed in water, lightly blotted and homogenized in 80% methanol at 0° C. Methanol was removed under vacuum and [^{14}C]ABA separated into free acid and 'bound' fractions (the latter presumed to be the glucosyl ester; Milborrow (1970)), by acid/base partitioning of the homogenate with dichloromethane (Zabadal 1974). Radioactivity in free acid and 'bound' ABA fractions was measured by scintillation counting as above.

Results

ABA Uptake. In a typical experiment, the endogenous ABA concentration of healthy tobacco leaves was 24 ± 2 ng g^{-1} fresh weight (mean ± standard error). Comparable, TMV-infected leaves contained 46 ± 6 ng g^{-1}. Endogenous ABA concentration varied with leaf age and environmental conditions: over all experiments the range was from 10–40 ng g^{-1} in healthy leaves and 20–80 ng g^{-1} in TMV-infected leaves. Infected leaves consistently contained between two and five times the ABA concentration of comparable healthy leaves sampled at the same time.

Leaf discs took up ABA from the culture medium for at least 24 h at a constant rate. After 24 h exposure to [^{14}C]ABA, 40% of the ABA taken up was recovered as the free acid and 60% was as the presumed glucosyl ester form, considered to be the major rapid-storage form of ABA (Milborrow 1970). This ratio of free to bound ABA was independent of exogenous ABA concentration.

Uptake of ABA varied somewhat from experiment to experiment; overall it depended strongly on ABA concentration in the medium over a very wide range (Fig. 1). Discs from TMV-infected leaves consistently took up more ABA than comparable discs from healthy leaves: over all experiments ABA uptake by infected discs was 3.3 ± 0.3 times (mean ± S.E.) that in healthy discs.

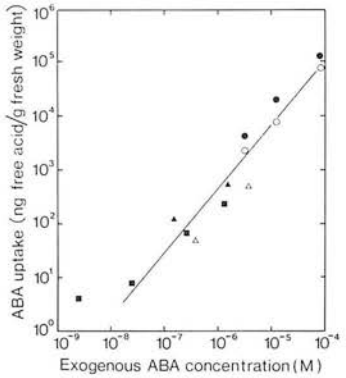

Fig. 1. Abscisic acid uptake by tobacco leaf discs. Discs were incubated for 24 h with various concentrations of ABA. Open symbols represent discs from healthy leaves; closed symbols discs from TMV-infected leaves. (■), cv. Samsun; (●, ○; ▲, △) two separate experiments with cv. White Burley

At exogenous ABA concentrations of around 10^{-7} to 10^{-6} M, sufficient ABA was taken up by discs to increase their internal ABA concentration by about two to six-fold after 24 h. This increase is similar in size to the increase in endogenous ABA stimulated by TMV infection.

Incubation of discs with higher exogenous ABA concentrations led to much bigger increases in disc ABA concentration: at 10^{-4} M exogenous ABA the level in discs rose by about 2,000-fold. This represents an ABA concentration in treated discs of almost 10^{-3} M, i.e. almost ten-fold higher than in the medium. ABA uptake cannot therefore have been solely by a passive diffusion process, but must have involved active uptake or sequestration after uptake.

Effects of ABA on RNA Synthesis. Rates of uridine uptake and incorporation by healthy leaf discs increased during culture (Fig. 2). ABA had little effect on uptake of uridine at 4 h or 24 h. Incorporation of uridine into cold-acid-insoluble material was significantly stimulated by ABA at both times of incubation. Maximum stimulation at 4 h was 3.5-fold, with $5 \cdot 10^{-5}$ M exogenous ABA. After 24 h, the maximum stimulation was 2-fold, with $4 \cdot 10^{-8}$ M exogenous ABA. Higher exogenous ABA concentrations caused smaller increases in incorporation.

The stimulation of precursor incorporation by ABA is confirmed by results shown in Fig. 3. Discs from TMV-infected leaves were pulse-labelled with [^{3}H]adenine after various times of incubation. Uptake and incorporation of adenine by control discs rose during culture. ABA began to inhibit uptake by 16 h, and reduced the rate of uptake by 60 to 70% after 46 h. Incorporation of adenine was significantly stimulated by ABA from 16 to 29 h; the higher the ABA concentration the earlier the stimulation. By 46 h, ABA apparently inhibited incorporation, but this

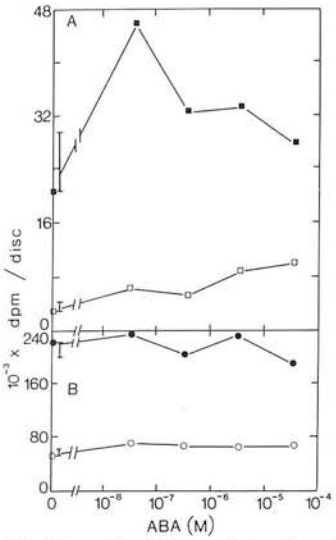

Fig. 2A and B. Effects of abscisic acid on [^{3}H]uridine incorporation (**A**) and uptake (**B**) by healthy tobacco leaf discs. Discs 6 mm in diameter of cv. Samsun leaves were cultured on medium with various concentrations of ABA, and labelled with 92.5 KBq ml^{-1} [^{3}H]uridine from 2–4 h (○, □) or from 22–24 h (●, ■). Vertical bars next to zero ABA points indicate least significant differences from the control at $P=0.05$

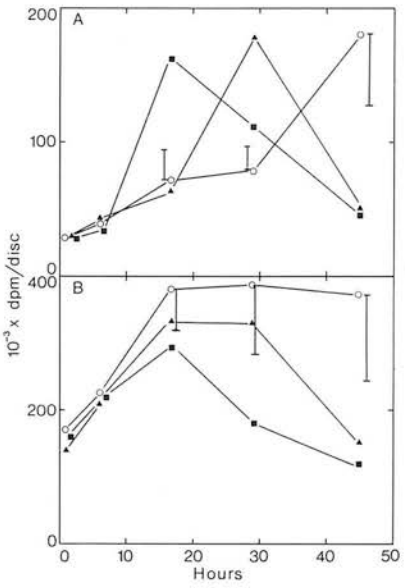

Fig. 3A and B. Effects of abscisic acid on [^{3}H]adenine incorporation (**A**) and uptake (**B**) by discs of TMV-infected tobacco leaf. Discs 6 mm in diameter from cv. Samsun leaves were cultured on medium with 0 (○); 10^{-6} M (▲) or 10^{-4} M (■) ABA and pulse-labelled with 92.5 KBq ml^{-1} [^{3}H]adenine for the last two hours before harvesting. Vertical bars indicate the least significant differences from the control (0 ABA) at $P=0.05$

could have been an indirect effect, resulting from the inhibition of adenine uptake by ABA at this time.

The data in Fig. 3 emphasise that the response to ABA varied strongly with time, probably because continuous ABA uptake caused continuously increasing ABA levels within discs.

Essentially similar results to those shown in Fig. 3 were obtained when [^{3}H]uridine was used as label for infected Samsun leaf discs, and also when healthy or infected discs of cv. White Burley were labelled with either [^{3}H]uridine or [^{3}H]adenine.

Figure 4 shows that TMV RNA and 25 S ribosomal RNA were labelled faster in discs treated with ABA than in control discs. In this experiment, uridine uptake was decreased by 15% by ABA. Thus the increased total incorporation shown in Figs. 2 and 3 was at least partly into high molecular weight RNAs, and both host and viral RNA syntheses were affected.

Effects of ABA on TMV RNA Accumulation. Table 1 shows that TMV RNA accumulation in two separate

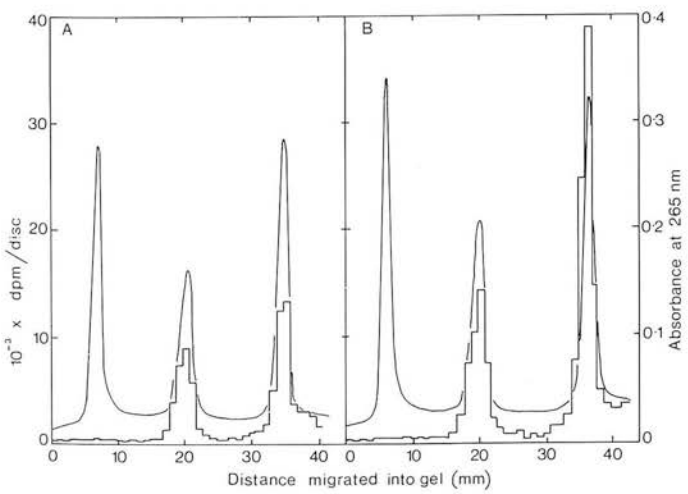

Fig. 4A and B. Polyacrylamide gel electrophoresis of [^{3}H]uridine labelled RNA from 15 mm diameter leaf discs of TMV-infected Samsun tobacco. Discs were incubated (**A**) without ABA or (**B**) with 10^{-6} M ABA for 24 h, and labelled for the entire period with 222 KBq ml^{-1} [^{3}H]uridine. DNA is at 6 mm, TMV RNA at 20 mm and 25 S ribosomal RNA at 36 mm into the gel. The continuous line shows absorbance at 265 nm; the histogram radioactivity. Each gel was loaded with one-eighth of the total nucleic acid extracted from four discs

Table 1. Changes in TMV RNA concentration in discs from cv. Samsun tobacco leaves incubated with various concentrations of abscisic acid

Experiment	Exogenous ABA (M)	Incubation time (h)	Increase in TMV RNA (μg g^{-1} fresh weight)
1[a]	0	24	13.2
	$4 \cdot 10^{-8}$	24	18.6
	$4 \cdot 10^{-7}$	24	19.4
	$4 \cdot 10^{-6}$	24	24.4
2[b]	0	72	28
	10^{-7}	72	82
	10^{-6}	72	65
	10^{-5}	72	150
	10^{-4}	72	80

[a] TMV RNA concentration at the start of incubation with ABA was 12 μg g^{-1} fresh weight

[b] TMV RNA concentration at the start of incubation with ABA was 18 μg g^{-1} fresh weight

experiments was enhanced at all levels of ABA treatment, and was between 1.4 and 5 times greater than in control discs over the time-interval measured. Balazs et al. (1973) reported increased infectivity in TMV-infected leaf discs treated with ABA. It is thus likely that the increased TMV RNA accumulation shown in Table 1 was accompanied by assembly into intact, infectious virus particles.

Discussion

By choosing an appropriate exogenous ABA concentration, it was possible to alter the ABA concentration of leaf discs by a physiologically reasonable amount, causing changes similar in magnitude to those induced naturally by TMV infection. However, we cannot exclude the possibility that ABA taken up by discs from the medium had a different intracellular distribution from that of endogenous ABA.

Small increases in ABA concentration within discs stimulated incorporation of uridine and adenine into host and viral RNAs. Stimulated incorporation was also found at high levels of ABA treatment, which raised disc ABA concentration to far beyond natural levels. The stimulation of incorporation was not because of any effect of ABA on precursor uptake, which was only altered by long incubations with high exogenous ABA concentrations.

Stimulation by applied ABA of precursor incorporation into TMV RNA was paralleled by greater net accumulation of TMV RNA. This suggests that ABA stimulation of precursor incorporation was due to an enhanced rate of synthesis, and not merely to an effect of ABA on the kinetics of precursor pool labelling.

It was not possible to demonstrate consistently a similar effect of ABA on ribosomal RNA accumulation. Leaves in the latter part of expansion growth begin to suffer a rapid net loss of ribosomal RNA (Fraser 1972). Discs incubated without ABA in our experiments either showed little change or net loss of ribosomal RNA. The high rate of turnover of ribosomal RNA may have obscured any effect of ABA stimulation of synthesis on ribosomal RNA content. TMV RNA in contrast is not turned over as leaves mature (Fraser 1973).

Our suggestion that ABA may increase the rate of RNA synthesis is contrary to several earlier studies showing inhibition. Many of these used embryonic tissues (Villiers 1968; Walbot et al. 1975) or actively dividing material (Pilet 1970; Minocha and DiBona 1979), whereas our tissue was mature and had virtually finished DNA synthesis and cell division (Fraser 1972). Possibly the developmental stage of a tissue affects its response to ABA.

Most of these reports did not measure changes in tissue ABA concentration, and may have been measuring inhibition of RNA synthesis at unnaturally high ABA concentrations. Bex (1972a) reported inhibition of RNA synthesis in coleoptiles only after uptake of more than 600 ng ABA g^{-1}.

Several reports of inhibition of RNA synthesis by ABA have dealt with ABA reversal of stimulation of RNA synthesis by another exogenously-applied hormone such as cytokinin (Nešković et al. 1977), gibberellic acid (Zwar and Jacobsen 1972) or auxin (Pilet 1970). In our experiments no other hormones were added.

Experiments reported by Nešković et al. (1977) indicated a possible stimulation of RNA synthesis: [^{32}P]orthophosphate incorporation into nucleic acids was stimulated by ABA in the presence of high concentrations of kinetin. Minocha (1979) found that ABA promoted cell division and DNA synthesis in cultured explants of Jerusalem artichoke tuber.

We do not know the mechanism by which ABA may stimulate RNA synthesis in tobacco leaves, and whether this is related to the means by which it inhibits RNA synthesis in other tissues. However, the results presented here suggest that small increases in ABA concentration might have a physiological significance in control of RNA synthesis. The increase in endogenous ABA concentration caused by TMV infection could therefore be at least partly responsible for the high rate of TMV RNA synthesis and early stimulation of host ribosomal RNA synthesis after infection (Fraser 1973).

We thank Su Loughlin for excellent technical assistance.

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Received 20 March; accepted 22 July 1980

Reprinted from *Physiological Plant Pathology* (1981) **18,** 267–278

Effect of systemic and local-lesion-forming strains of tobacco mosaic virus on abscisic acid concentration in tobacco leaves: consequences for the control of leaf growth

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(*Accepted for publication December 1980*)

Abscisic acid (ABA) is a plant hormone which inhibits growth. Its concentration increased 2- to 6-fold in leaves of White Burley tobacco systemically-infected with the *vulgare* strain of tobacco mosaic virus (TMV). The concentration of "bound" ABA (presumed to be the glucosyl ester) was not affected by infection. TMV strain *flavum* produced necrotic lesions and localized infection. Commencing at the time of lesion appearance, free ABA increased by up to 18-fold, and "bound" ABA by up to 3-fold. The increase in ABA occurred in or very close to the lesions. ABA concentration also increased in uninfected leaves of *flavum*-infected plants, probably by ABA transport from inoculated leaves. The means by which systemic and localized TMV infection might stimulate ABA synthesis are discussed.

Virus infection reduced leaf growth. Healthy plants were sprayed with ABA to test whether growth inhibition could be caused by the raised ABA concentration after infection. Both infection and application of ABA to healthy plants decreased leaf growth by inhibiting cell division: neither affected cell expansion. The 2 treatments had similar effects when applied at different stages of leaf development. Quantitatively, raising the ABA concentration of healthy leaves by an amount similar to the increase caused by TMV gave a similar inhibition of leaf growth. It is concluded that raised ABA concentration after infection is a major cause of the inhibited growth of infected plants.

INTRODUCTION

Virus infection of plants frequently inhibits growth and causes changes similar to senescence. These changes may in part result from virus-induced changes in plant hormone concentrations. Abscisic acid (ABA) is a naturally occurring hormone which inhibits growth and, if supplied exogenously, promotes senescence [reviewed in *13*]. Although there have been reports of altered ABA concentrations after virus infection [*1, 3, 18*], there has been no detailed investigation of changes in ABA concentration during various types of host–virus interaction. Nor has there been any assessment of the effects of virus-induced changes in ABA on plant growth.

In this paper we report changes in ABA in White Burley tobacco after infection with tobacco mosaic virus (TMV). This host was chosen because it allows comparison of 2 different host reactions to infection. With TMV strain *vulgare* infection is systemic, while with strain *flavum*, the virus is restricted to necrotic local lesions which form around the sites of infection.

The importance of virus-induced changes in endogenous ABA in control of plant growth was examined: the effects of infection on plant growth were compared with those caused by artificially increasing the ABA concentration in healthy plants.

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MATERIALS AND METHODS

Plants and viruses

Tobacco plants (*Nicotiana tabacum* L.) cv. White Burley were grown in John Innes No. 2 compost in 12·5 cm pots. Plants were kept in a glasshouse under natural lighting conditions. The temperature was 16 °C at night and 20 to 25 °C during the day.

TMV strains *flavum* and *vulgare* were multiplied in the systemic host *N. tabacum* cv. Samsun. Inocula were prepared by grinding frozen, infected leaves in a pestle and mortar and diluting 10^2 to 10^5 times with 50 mM sodium phosphate buffer, pH 7·0. White Burley plants with 7 to 9 expanded leaves were lightly dusted with Carborundum on 2 lower leaves and inoculated by rubbing with virus suspension. Control plants were rubbed with sterile phosphate buffer alone (sham-inoculated) or were untreated. Leaves were washed with running tap water immediately after inoculation.

TMV multiplication

Virus multiplication was followed by measuring changes in TMV RNA content. Total nucleic acid was extracted from leaf samples (0·5 to 1·0 g fresh wt) by a phenol-detergent procedure and purified as described previously [*7*]. TMV RNA content was measured by polyacrylamide gel electrophoresis [*10*]. Each assay of TMV RNA was performed in duplicate.

ABA determination

ABA occurs in leaves as the free acid and in a "bound" form, presumed to be ABA glucosyl ester [*12*]. To measure the concentrations of free and "bound" ABA, 1 g leaf samples were homogenized in 80% acetone at 0 °C. Occasionally 80% methanol was used as extractant when the concentration of free ABA only was required. Both solvents contained 10 mg l^{-1} butylated hydroxytoluene as antioxidant. ^{14}C-ABA (0·5 nCi; 11 ng of ±)-*cis-trans*-[2-^{14}C]-ABA, 11·9 mCi mmol^{-1}; Radiochemical Centre, Amersham) was added to the homogenates to allow measurement of ABA recovery and to facilitate location of ABA on chromatograms.

The organic solvent was removed under vacuum, and free and "bound" ABA fractions prepared by acid/base partitioning with dichloromethane [*24*]. Free ABA was removed in the acid dichloromethane fraction, leaving "bound" ABA in the aqueous residue. Methyl abscisate (MeABA) formed from "bound" ABA in methanolic extracts [*15*] was quantitatively removed in the alkaline dichloromethane fraction and did not interfere with the assay of free ABA using methanol extraction.

A further 0·5 nCi of [^{14}C]-ABA was added to the aqueous residue from dichloromethane partitioning, and ABA released from the "bound" form by alkaline hydrolysis [*12*] then re-extracted with dichloromethane at pH 3·0.

Free ABA and ABA released from the "bound" form (referred to as "bound" ABA) were purified by thin-layer chromatography (t.l.c.) on silica gel developed with toluene : ethyl acetate : acetic acid (25 : 15 : 2, v/v). *Cis-trans* ABA was located on chromatograms in a radiochromatogram scanner (Birchover Instruments Ltd.) and eluted with methanol. Free and "bound" ABA were methylated with ethereal diazomethane [*19*] and MeABA further purified by t.l.c. on silica gel developed with

either hexane : ethyl acetate (3 : 1, v/v) or chloroform : ethyl acetate (9 : 1, v/v). MeABA was located in the scanner and eluted with ethyl acetate.

Total MeABA content of samples was measured by gas chromatography using a 2 m column of 2·5% OV-1/Gas Chrom Q at 235 °C; injector and detector temperatures were maintained at 300 °C. The carrier gas was argon : methane (95 : 5) at a flow rate of 30 ml min^{-1} through the column. ABA was measured using an electron capture detector [*20*], calibrated by chromatography of known amounts of MeABA (^{14}C-ABA from the Radiochemical Centre, methylated as above). The identification of MeABA on chromatograms was confirmed by combined gas chromatography–mass spectrometry.

Recovery of [^{14}C]-ABA was measured by liquid scintillation counting of a sample of the ethyl acetate eluate in toluene containing 5 g l^{-1} 2-(4′-*t*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole. Correction of counts to 100% counting efficiency was by the external standard channels-ratio method. The typical recovery of [^{14}C]-ABA in purified samples was 60%. Leaf ABA concentrations measured by gas chromatography were corrected to 100% recovery using the [^{14}C]-ABA recovery measurements. The weight of [^{14}C]-ABA present was subtracted from the total.

ABA assays were performed on duplicate samples taken from a minimum of 4 plants at each harvest. All experiments were repeated at least once. The variation in ABA concentration between replicate samples was low: all individual determinations were within ±5% of the mean.

Leaf area and cell number

Leaf areas were measured with a photoelectric digital scanner [*5*]. Leaf cell number was determined by macerating leaf discs in 5% chromic acid [*4*] and counting samples in a haemocytometer slide. Cell fresh weights were obtained by dividing leaf fresh weight by the calculated number of cells per leaf.

Leaf diffusive resistance

Diffusive resistance as a measure of stomatal aperture in leaves was meaured with an automatic porometer (Delta T Devices, Cambridge).

RESULTS

ABA in vulgare-*infected plants*

The concentration of free ABA in leaves of healthy plants varied considerably, within the range 7 to 42 ng g^{-1} (Fig. 1, Table 1). These values agree well with other published estimates for unstressed tobacco leaves [*3*, *18*]. No consistent differences were found between sham-inoculated and untreated control leaves.

While there was some tendency for expanding leaves to have higher free and "bound" ABA concentrations than mature leaves, it is likely that some of the variation in ABA concentration was caused by changes in environmental conditions between and during experiments. To minimize the effects of such environmental changes, healthy and infected plants were grown together and samples from both were always taken at the same time.

Most of the ABA in healthy leaves was in the "bound" form; depending on leaf age, free ABA constituted only 7 to 21% of the total. High proportions of "bound" ABA have also been reported for unstressed cotton and vine leaves [*8*, *11*].

Accumulation of TMV RNA was detectable in both inoculated and systemically-infected leaves by 5 days after inoculation, and continued for a further 20 to 25 days (Fig. 1). TMV infection had little effect on the "bound" ABA concentration in

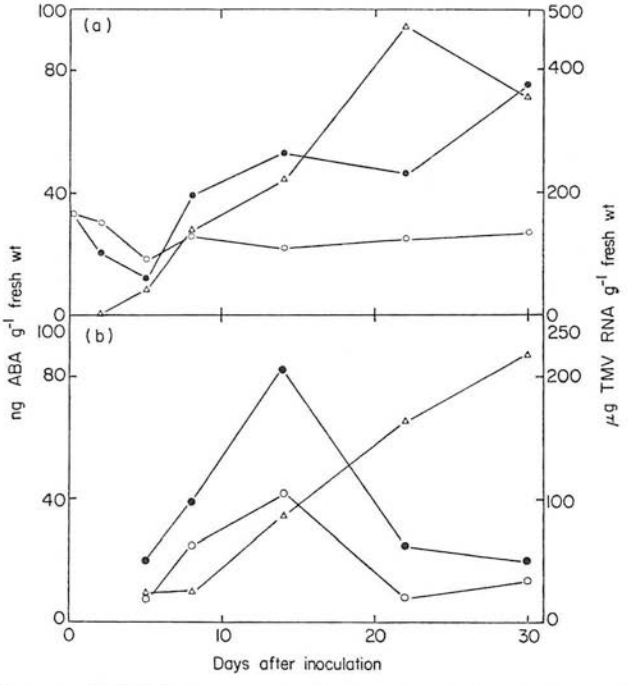

FIG. 1. ABA and TMV RNA concentration in *vulgare*-infected tobacco leaves. Plants were inoculated with TMV strain *vulgare* diluted $1 : 2 \times 10^2$ (●) or with buffer (○). Free ABA concentration was compared in (a) inoculated leaves and in (b) the 3 leaves above the upper inoculated leaf. (△) TMV RNA concentration.

TABLE 1

Concentration of free and "bound" ABA in healthy and vulgare*-infected tobacco leaves*[a]

	Leaf	Free ABA ng g^{-1}	"Bound" ABA ng g^{-1}	Total ABA ng g^{-1}	Free ABA % total
Infected	5	33	145	178	19
	9	50	106	156	32
	13	73	38	111	66
Healthy	5	8	115	123	7
	9	8	88	96	8
	13	12	45	57	21

[a]Plants were inoculated on leaves 5 and 6, numbered from the base of the plant, with TMV strain *vulgare* diluted $1 : 2 \times 10^2$ or were untreated. Free and "bound" ABA concentrations were compared in leaves of different ages 30 days later.

leaves, but increased the free ABA concentration to between 2 and 6 times that in comparable healthy leaves (Table 1, Fig. 1). Except for the very early stages of infection, the TMV-stimulated increase in free ABA concentration was present throughout the virus accumulation period, and occurred in both inoculated and systemically-infected leaves.

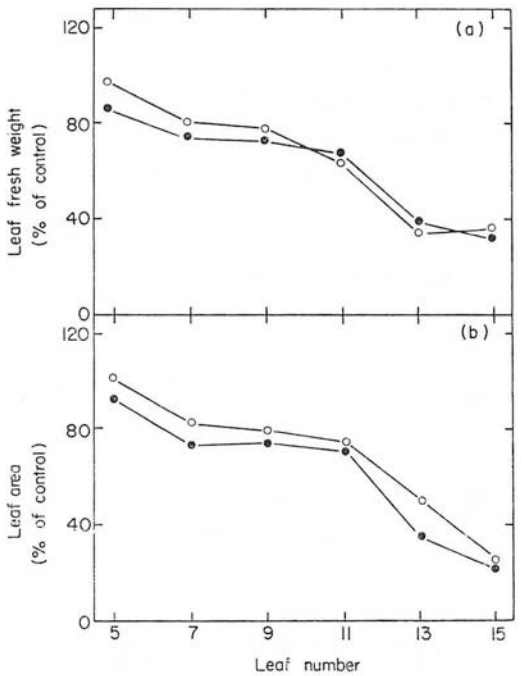

FIG. 2. Effect of ABA and *vulgare* infection on leaf growth. Plants were either inoculated with TMV strain *vulgare* diluted $1 : 2 \times 10^2$ (●) or were sprayed with a 5×10^{-5} M solution of (±)-*cis-trans* ABA (○). (a) Leaf fresh weight and (b) leaf area were measured 30 days later. Leaf area and weight were expressed as percentages of untreated controls. Leaves were numbered from the base of the plant.

Effect of exogenous ABA and vulgare *infection on leaf growth*

Infection with *vulgare* reduced leaf growth (Fig. 2 and ref. [7]). To test how much of the growth inhibition was due to the raised concentration of ABA in infected plants, healthy plants were sprayed with ABA on alternate days and their growth compared with that of infected plants. Growth parameters were measured after 30 days infection or ABA treatment. Leaves at higher positions on the plant had become exposed to infection or ABA at progressively earlier stages of development.

Spraying with 5×10^{-5} M (±)-*cis-trans* ABA reduced leaf growth (measured as fresh weight or area) by an amount similar to TMV infection [Fig. 2(a), (b)]. The reduction in growth by either treatment depended on leaf age: the younger the leaf at infection or start of ABA treatment, the greater the inhibition of growth. TMV infection and ABA treatment had reduced shoot height by 50 or 32% respectively after 30 days.

Figure 3 shows that the reduction of leaf area by TMV infection or ABA treatment was correlated with reduced cell number per leaf. This relationship held for leaves infected or ABA-treated at various stages of development, and thus showing a wide range of growth inhibition. The mean cell fresh weights in ABA-treated and *vulgare*-infected leaves were 102 ± 10 and $116 \pm 7\%$ respectively of the mean cell fresh weight in healthy control plants; thus neither treatment inhibited cell expansion.

The effect of spraying with ABA on the internal concentration of ABA was measured during the growth period. After 8 days ABA treatment, leaves were thoroughly rinsed with alcohol and water to remove surface ABA. The internal concentration of free ABA in ABA-sprayed leaves was 65 ± 20 ng g^{-1} higher than in comparable leaves of water-sprayed healthy control plants. Thus the increase in ABA concentration caused by spraying was of a similar size to that caused by TMV infection.

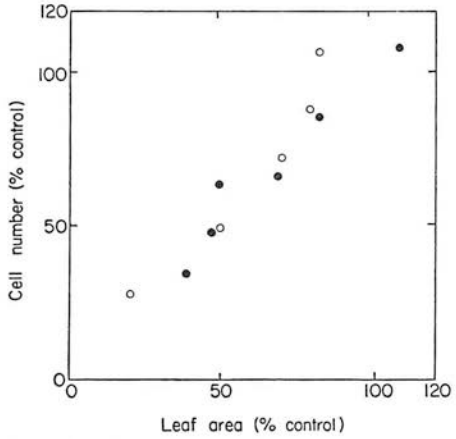

FIG. 3. Effect of ABA and *vulgare* infection on leaf cell number. Plants were treated as in Fig. 2. Leaf cell number and area in infected (●) and ABA-treated (○) leaves were expressed as a percentage of control plants and were plotted for leaves of different ages at different positions on the stem.

ABA in flavum*-infected plants*

Most *flavum*-infected plants showed only necrotic lesions in inoculated leaves with no systemic spread of virus. Occasionally, individual plants showed severe systemic symptoms, probably caused by virus mutants [6]; such plants were discarded.

In the experiment shown in Fig. 4, leaves were inoculated with a high virus concentration, producing an average 4000 lesions per leaf. Accumulation of TMV RNA was detectable by the time of lesion appearance, 2 to 3 days after inoculation. Lesion size and TMV RNA content increased up to 8 days. After this, leaves suffered necrotic collapse because of the high lesion density.

The concentration of free ABA in inoculated leaves increased at about the time of lesion appearance [Fig. 4(a)]. In several repeat experiments no consistent evidence was found for any increase in ABA before lesion appearance. ABA concentration (as ng g^{-1} fresh wt) in infected leaves continued to increase until 8 days after inoculation

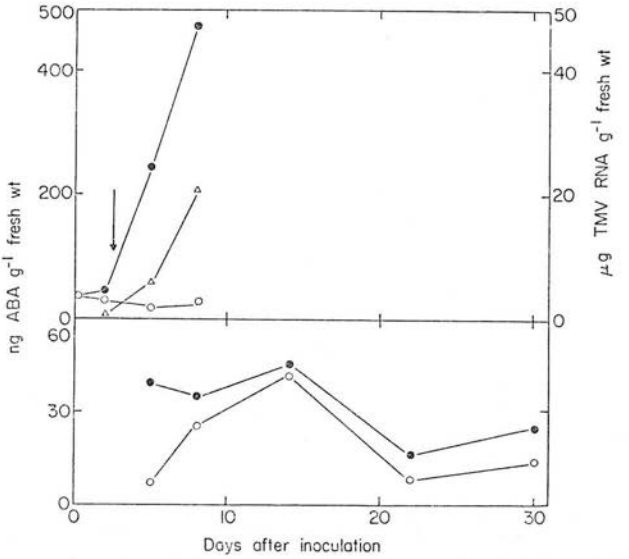

FIG. 4. ABA and TMV RNA concentration in *flavum*-infected tobacco plants. Plants were inoculated with TMV strain *flavum* diluted $1 : 2 \times 10^2$ (●) or with buffer (○). Free ABA concentration was compared in (a) inoculated leaves and in (b) the 3 leaves above the upper inoculated leaf. (△) TMV RNA concentration in *flavum*-inoculated leaves. The arrow indicates the approximate time of lesion appearance.

TABLE 2

Concentration of free and "bound" ABA in healthy and flavum*-infected tobacco leaves*[a]

	Free ABA ng g^{-1}	"Bound" ABA ng g^{-1}	Total ABA ng g^{-1}	Free ABA % total
flavum-inoculated leaf				
12 h before lesion appearance	33	79	112	29
sham-inoculated leaf	27	95	122	22
flavum-inoculated leaf 144 h				
after lesion appearance	138	182	320	43
sham-inoculated leaf	21	57	78	27

[a]Plants were inoculated with either TMV strain *flavum* diluted $1 : 2 \times 10^2$ or with buffer (sham-inoculated). Lesions (1500 per leaf) appeared approximately 84 h after inoculation.

it was 18 times higher than in comparable leaves of healthy plants. Losses in leaf fresh weight caused by extensive necrotization contributed some but not all of the increase in ABA concentration. Eight days after infection, *flavum*-inoculated leaves contained 12 times more free ABA per leaf than healthy leaves. *Flavum* infection also increased the concentration of "bound" ABA in inoculated leaves (Table 2). After lesion appearance the concentration was 3 times that in healthy leaves. Thus *flavum* infection had less effect on "bound" than free ABA concentration.

In the experiment shown in Fig. 5, leaves were inoculated with a low virus concentration, producing an average of 25 well-separated lesions per leaf. Discs bearing a single lesion with a 1 to 2 mm thick ring of non-necrotic tissue were cut at

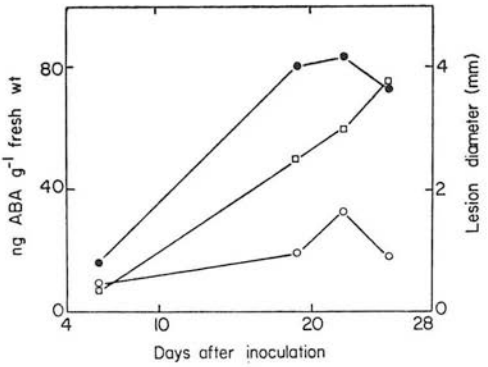

FIG. 5. ABA concentration in lesions. Plants were inoculated with TMV strain *flavum* diluted 1 : 10^5. Free ABA concentration in leaf discs 4 to 7 mm in diameter bearing single lesions (●) was compared to that in similar discs of healthy tissue from the same leaf (○). Fifty to 400 discs were pooled for each determination. (□) Lesion diameter in mm.

various times after inoculation. The concentration of free ABA in such discs increased as lesions expanded. Discs bearing lesions had 2 to 4·3 times the ABA concentration of control discs cut from non-lesion areas of the same leaf. Thus the increase in ABA concentration in leaves inoculated with *flavum* was associated with the lesions rather than being a response of the leaf as a whole to infection. The increase in leaf ABA concentration was also proportional to the number of lesions formed per leaf, over a very wide range (data not shown).

Non-infected leaves of *flavum*-infected plants contained a higher concentration of free ABA than comparable leaves of healthy plants [Fig. 4(b)], although ABA concentrations were lower than in inoculated leaves. Five days after inoculation of the lower leaves the ABA concentration in non-infected leaves was 6 times that in healthy plants. After the necrotic collapse of the inoculated leaves at 8 days, the ABA concentration in non-infected leaves remained consistently 1·1 to 2 times higher than in healthy plants up to 30 days.

Transport and metabolism of ABA in flavum-*infected plants*

The increase in ABA concentration in upper, non-infected leaves may have been due to enhanced synthesis within these leaves, or to transport of ABA from the lower, inoculated leaves. To test the latter possibility, [^{14}C]-ABA was applied to inoculated leaves soon after the appearance of lesions and the distribution of labelled ABA followed in the plant (Table 3).

After 24 h, 50 to 60% of the [^{14}C]-ABA had been metabolized to the "bound" form, considered to be the major rapid storage form [*13*]. Detectable transport of [^{14}C]-ABA had occurred to other parts of the plant. The free acid [^{14}C]-ABA concentrations in the shoot tip and first untreated leaf were 0·9 and 3·1% of the free acid [^{14}C]-ABA concentration in the 2 treated leaves. This transport of [^{14}C]-ABA to uninfected parts of the plant can be used to approximate the amount of ABA in the non-infected leaves which originated in the lower, inoculated leaves. It is assumed that the metabolism and transport of applied ABA is the same as that of endogenous ABA.

TABLE 3

Transport and metabolism of ^{14}*C-ABA in* flavum*-infected plants*[a]

		Time after ABA treatment		
	Leaf	24 h	66 h	96 h
		10^{-2} d min^{-1}/leaf		
Free ABA	1	456	378	336
	2	14	21	31
		(3·1%)	(5·6%)	(9·2%)
	3	4	5	3
		(0·9%)	(1·3%)	(0·9%)
"Bound" ABA	1	678	514	886
	2	14	21	35
	3	4	3	3

[a]Plants were inoculated on 2 lower leaves with TMV strain *flavum* diluted 1 : 10^3. After lesion appearance, each inoculated leaf was brushed with 2 ml of a $1{\cdot}2 \times 10^{-4}$ M solution of (±)-*cis-trans* [^{14}C]-ABA (specific activity 1·8 mCi mmol^{-1}). At various times after treatment leaves were washed with alcohol and water to remove surface ABA and free and "bound" ABA extracted and purified by t.l.c. The radioactivity present in free and "bound" forms was measured in (1) both treated leaves, in (2) the first leaf above the upper inoculated leaf and in (3) the shoot tip. Figures in parentheses are percentages of the free [^{14}C]-ABA present in both treated leaves.

From Fig. 4 the amount of ABA in the 2 inoculated leaves 4 days after infection was approximately 1800 ng. If 3·1% (55 ng) was transported to the first uninoculated leaf (fresh weight 1·8 g) within 24 h, this would produce an increase in free ABA of 30 ng g^{-1} which is close to the increase observed. This calculation takes no account of possible degradation of ABA in the upper leaf, but shows that the amount of transport is of the correct order of magnitude to explain the observed increase in free ABA concentration in non-infected leaves [Fig. 4(b)].

DISCUSSION

Several earlier studies have measured changes in concentrations of plant hormones after virus infection. Growth inhibitor levels, measured by bioassay, were increased in lupins infected with pea mosaic virus [*21*]. and in rice seedlings infected with rice tungro virus [*17*]. Using physical methods for identification and measurement of ABA, Rajagopal [*18*] found decreased ABA in TMV-infected tobacco, but examined only the very early stages of systemic infection. Bailiss [*2*] found no alteration in ABA concentration in cucumber shoots infected with cucumber mosaic virus, while Bailiss *et al.* [*3*] reported an increase in ABA concentration in leaves of Xanthi-nc tobacco forming lesions after TMV infection.

Our results show that the concentration of free ABA in tobacco leaves was increased 2- to 6-fold on systemic infection with TMV, and that the increase persisted for the duration of virus multiplication. Infection had little effect on "bound" ABA concentration: the increase in free ABA was probably therefore a result of enhanced synthesis and not of release from the "bound" form. Infection with TMV strain *flavum* caused increases in both free and "bound" ABA concentration, which were

associated with necrotic lesion formation. The increase in free ABA concentration (up to 18-fold) was greater than the increase in "bound" ABA concentration. ABA concentration was also increased in the upper, non-infected leaves of *flavum*-infected plants; this increase could be largely explained by transport of ABA from the lower, inoculated leaves.

Although *flavum* stimulated a much greater increase in ABA concentration than *vulgare*, it accumulated to much lower concentration (20 μg TMV RNA g^{-1}) than *vulgare* (400 μg g^{-1}). The increase in ABA was therefore not related to the amount of virus made. The results suggest that virus infection might cause altered ABA concentration in two separate ways: by causing necrosis, and by some mechanism related to TMV multiplication in non-necrotizing tissue. Necrosis may cause water loss, and water deficit induces large and rapid increases in ABA concentration [*23*]. The means by which TMV multiplication in non-necrotic tissue might increase ABA synthesis are less clear. One possibility is by an effect on chloroplasts, the major site of ABA synthesis [*13*]. Increased chloroplast membrane permeability to ABA is thought to enhance ABA synthesis within the chloroplast [*14*]. and TMV infection has been reported to alter the properties of chloroplast membrane proteins [*9*].

Systemic infection with TMV strain *vulgare* severely inhibited leaf growth. In *flavum*-infected plants, growth of uninfected leaves is also inhibited [*6*]. Our results suggest that these effects of infection on growth could be consequences of the raised ABA levels after infection.

Qualitatively, systemic TMV infection and repeated application of ABA to healthy plants both reduced leaf growth by inhibiting cell division, rather than cell expansion. Both treatments had similar effects when applied at various stages of leaf development. Our finding that ABA is transported from infected to non-infected parts of plants inoculated with strain *flavum* provides a possible explanation of the reduced growth of non-infected leaves [*6*].

Quantitative evidence that the increase in ABA concentration caused by TMV infection is capable of producing the observed inhibition of growth is more difficult to obtain. Exogenously applied ABA may have a different intracellular distribution and metabolism from endogenous ABA. Furthermore, it is experimentally difficult to apply exogenous ABA so as to mimic exactly changes in ABA induced by virus infection over an extended growth period. Our approach was to spray frequently throughout the growth period with a comparatively low concentration of ABA, attempting to maintain a raised concentration and replace ABA which was metabolized or sequestered. Our results indicate that when the ABA concentration of healthy plants was raised by an amount similar to the increase caused by TMV infection, the inhibition of growth was similar in size to that caused by TMV infection. This suggests that the virus-induced increase in ABA was likely to be a major factor in the inhibition of leaf growth. It does not exclude the possibility that virus-induced changes in other hormones could also be important [cf. refs. *1*, *2*], or that the large accumulation of viral nucleoprotein in a systemic infection may have significantly reduced host growth by competition for metabolites.

ABA is important in control of stomatal closure [*16*]. However, it is unlikely that the enhanced ABA levels in TMV-infected plants caused reduced growth by closing stomata hence inhibiting gas exchange. We found no difference in stomatal aperture,

measured as diffusive resistance, between healthy leaves ($2{\cdot}2 \pm 0{\cdot}1$ s cm^{-1}) and leaves systemically-infected with TMV ($2{\cdot}5 \pm 0{\cdot}1$ s cm^{-1}).

Virus-induced increase in leaf ABA concentration may have produced a situation favourable for virus multiplication. Inhibition of host growth would reduce competition for nucleoside triphosphates and amino acids required for virus synthesis. We have reported elsewhere that application of ABA to tobacco leaf discs increases the rate of TMV RNA accumulation [*22*].

We thank Babinder Sandhar and Su Loughlin for excellent technical assistance and John Eagles of the Food Research Institute, Norwich for g.c.–m.s. authentication of ABA.

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Printed in Great Britain by Henry Ling Ltd., at the Dorset Press, Dorchester, Dorset

Reprinted from *Physiological Plant Pathology* (1981) **19,** 69–76

Evidence for the occurrence of the "pathogenesis-related" proteins in leaves of healthy tobacco plants during flowering

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(*Accepted for publication April 1981*)

Four host-coded "pathogenesis-related" proteins accumulate in local-lesion-forming varieties of tobacco after infection with tobacco mosaic virus. It has been suggested that they are involved in the acquired systemic resistance of plants to a second inoculation.

It is shown that while these proteins were undetectable in leaves of young, healthy plants of the cv. Xanthi-nc., they did accumulate in large amounts when healthy plants flowered. Accumulation depended on the presence of both the developing inflorescence and the senescing lower leaves. The 2 commonest of these proteins from flowering, healthy plants were shown to behave identically to analogous proteins from young, infected plants on gel filtration, ion-exchange chromatography, and electrophoresis under denaturing and non-denaturing conditions.

When healthy plants were experimentally treated so that they accumulated very different amounts of "pathogenesis-related" proteins, no correlation was found between protein concentration and susceptibility to tobacco mosaic virus infection, measured either as lesion number or size. The role of the "pathogenesis-related" proteins in acquired systemic resistance is therefore questioned.

INTRODUCTION

The tobacco cultivars Xanthi-nc. and Samsun NN form necrotic local lesions after infection with tobacco mosaic virus (TMV). At least 4 new, host-coded proteins are detected after infection, in both inoculated and non-inoculated parts of the plant [*6, 8, 12, 14, 16*]. Antoniw *et al.* [*2*] suggested that they be named "pathogenesis-related" proteins (PR proteins).

TMV infection of hypersensitive cultivars of tobacco induces an apparent systemic resistance, measured as a decrease in size and number of lesions formed after a second, challenge inoculation [*9, 13, 15*]. Treatment of healthy plants with poly-acrylic acid similarly induces resistance to a challenge inoculation with TMV, and also causes production of PR proteins [*7*]. These results have led to suggestions that the PR proteins may be part of an inducible, antiviral defence mechanism [*7, 8, 14*]. However, attempts to demonstrate antiviral activity of PR proteins directly do not seem to have been successful [*8, 9*].

The PR proteins are generally considered to be absent from healthy, untreated plants [*2, 6, 14, 16*]. In this paper I report that while PR proteins are normally undetectable in healthy plants, they do accumulate in large amounts in untreated plants at the onset of flowering.

0048–4059/81/040069+08 $02.00/0

MATERIALS AND METHODS

Plants and virus

Tobacco plants (*Nicotiana tabacum* L. cv. Xanthi-nc.) were grown in a glasshouse and TMV tomato strain 0 was multiplied and purified as previously described [*5*]. Xanthi-nc. leaves were inoculated by rubbing with a suspension of TMV at 1 to 5μg ml^{-1} in 10 mM sodium phosphate buffer, pH 7, using Carborundum as abrasive. Lesions were counted 7 to 10 days later. Lesion diameters were measured using a ×50 stereoscopic microscope. Chlorophyll was extracted from 1 g leaf samples into 80% acetone and measured spectrophotometrically [*17*].

Protein extraction and purification

Leaf samples (5 g) were ground at 0 °C in 5 ml phosphate–citrate buffer, pH 2·8, containing mercaptoethanol [*1*]. The homogenate was filtered through muslin and centrifuged at 10 000 ***g*** for 20 min. The supernatant formed the crude extract.

PR proteins were partially purified from 20 ml batches of crude extract by gel filtration on a 140×0·8 cm column of Sephadex G50, followed by ion exchange chromatography on a 12×0·8 cm column of DEAE cellulose (DE 52, Whatman). Buffers and fractionation conditions were as described by Antoniw *et al.* [*2*]. Fractions of the DEAE column effluent were monitored for absorbance at 280 nm; fractions containing protein peaks were concentrated by dialysis against solid sucrose.

Electrophoresis

Sucrose was added to crude extracts to a final concentration of 5%. Samples of 10 to 500 μl of crude extract, or of partially purified PR proteins, were fractionated by electrophoresis on 10% polyacrylamide gels under non-denaturing conditions. The gels were 75 mm long, 6 mm diameter; buffers were as described by Davis [*4*]. Electrophoresis was for 5 h at 3 mA per gel constant current, at 4 °C.

Further samples of extract or partially-purified PR proteins were adjusted to neutrality with 1 M NaOH, then mixed with an equal volume of buffer consisting of 120 mM Tris–HCl pH 6·8; 4% sodium dodecyl sulphate (SDS) and 10% 2-mercapto-ethanol. The mixture was heated in a boiling water bath for 3 min to denature proteins, then fractionated on denaturing (SDS) gels using the buffer system of Laemmli [*11*]. The stacking gel was 5% and the separating gel 12% acrylamide. Electrophoresis was for 7 h at 3 mA per gel constant current, at 20 °C.

After electrophoresis, gels of both types were stained overnight in 0·1% PAGE Blue G90 in 40% methanol, 7% acetic acid, then destained in 14% methanol, 7% acetic acid. Destained gels were stored in 7% acetic acid. Mobilities of protein bands were expressed relative to that of the bromophenol blue marker run in each gel (R_F).

Gels were scanned for absorption at 260 nm in a Joyce-Loebl gel scanner; PAGE Blue G90 has an absorption maximum at this wavelength. On the scans, the peak area of a protein band was shown to be proportional to the weight of protein in the band, by electrophoresis of different amounts of the same extract. However, intensity of staining varied from experiment to experiment. To allow quantitative comparisons between experiments, samples of a standard crude extract were fractionated with each batch of gels. The standard extract was prepared from expanded leaves on

plants 20 cm tall. The leaves had an average of 50 lesions and were extracted 6 days after inoculation with TMV at 1 μg ml^{-1}. The concentration of the commonest PR protein (PR1a) [2] was defined as 100 units g^{-1} fresh wt; concentrations of all other PR proteins were expressed relative to this. The limit of detection, set by considerations of gel loading and scanner sensitivity, was 0·05 units g^{-1}.

RESULTS

PR proteins in TMV-infected leaves

Figure 1 shows fractionation on non-denaturing gels of proteins extracted from expanded leaves of young (20 cm tall) plants. Proteins extracted from infected leaves

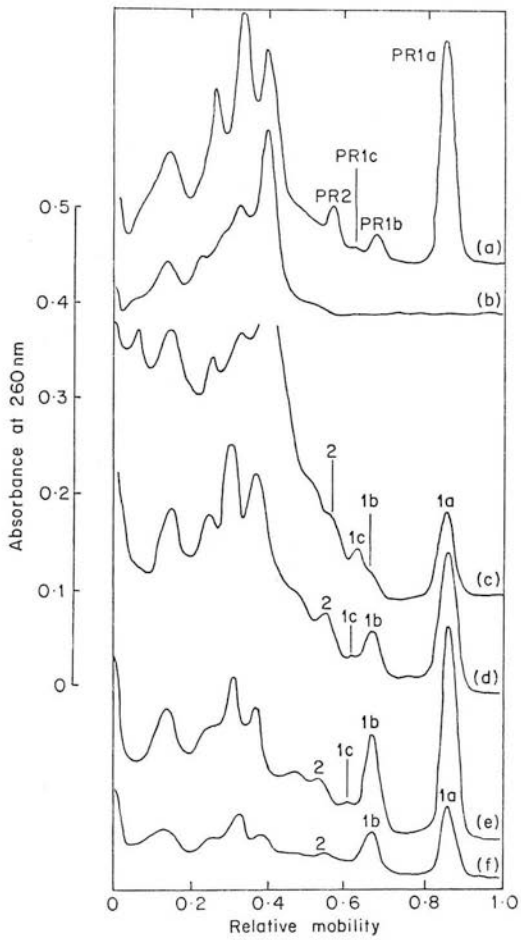

FIG. 1. Electrophoresis on non-denaturing polyacrylamide gels of tobacco leaf protein extracts. (a) From leaves of young plants with a mean of 50 lesions per leaf, 6 days after inoculation with TMV at 1 μg ml^{-1}. (b) From leaves of young, healthy plants, 6 days after sham inoculation with carborundum and sterile phosphate buffer. (c) to (f) From leaves of healthy, untreated, mature plants, which were flowering and on which the lower leaves were senescing. Leaf samples were taken from (c), 900–1000 mm; (d), 600–700 mm; (e), 250–350 mm; and (f), 0–150 mm high on plants.

showed four highly mobile bands [Fig. 1(a)]; these bands were absent from healthy leaf extracts [Fig. 1(b)]. The infected leaf bands are identified as pathogenesis-related proteins PR1a, PR1b, PR1c and PR2 in order of decreasing mobility, and using the nomenclature system proposed by Antoniw *et al.* [2]. Their respective R_F values were 0·86, 0·69, 0·59 and 0·53. These values compare well with those found by Antoniw *et al.* [2] for 10% gels, i.e. 0·87, 0·69, 0·59 and 0·55.

The 4 PR bands were also detected in non-inoculated leaves of infected plants. Between 10 and 15 days after inoculation of one-half of each of 3 lower leaves, the concentration of PR1a in the uninoculated opposite halves ranged from 16 to 64 units g^{-1}. In uninoculated upper leaves it was from 3·5 to 7 units g^{-1}.

PR proteins in healthy plants

Figure 1(c) to (f) shows gels of proteins extracted from leaves of older (120 cm tall) plants. These plants had received no virus inoculation or experimental treatment; they were in flower and the lower leaves were yellow. The plants showed no symptoms of disease, whether viral, fungal or bacterial, and examination of sap in the electron microscope revealed no virus particles.

PR proteins 1a 1b and 2 were detected in all ages of leaf (Table 1). The highest concentrations of PR1a and PR1b were in leaves on the lower half of the plant, which

TABLE 1

Concentrations of pathogenesis-related proteins and chlorophyll in leaves of uninfected, mature Xanthi-nc. tobacco plants[a]

Height of leaves on plant (mm)	Chlorophyll (mg g^{-1})	Pathogenesis-related protein (relative units g^{-1})[b] PR1a	PR1b	PR1c	PR2
900–1000	0·70	10·3	trace[c]	2·5	trace
600–700	0·30	16·6	3·6	trace	2·4
250–350	0·11	19·5	8·5	trace	1·5
0–150	0·02	9·8	3·8	nd[d]	1·2

[a] Plants were 1200 mm tall and flowering.

[b] Expressed relative to the concentration of PR1a (100 units g^{-1}) in leaves of young Xanthi-nc. plants with an average of 50 lesions per leaf, 6 days after inoculation with TMV.

[c] Indicates a band visible to the eye, but not sufficiently resolved on the scan for area measurement.

[d] Not detectable as a band by eye.

had lost most of their chlorophyll. PR1c was found more in the upper part of the plant. Generally, the relative proportions of the four PR proteins were similar to those in infected leaves, with PR1a present at the highest concentration, and PR1c and PR2 at the lowest.

In the oldest leaves on the plant, the PR proteins formed the major components of the proteins extracted at pH 2·8. The less mobile, acid-soluble proteins found in younger leaves [Fig. 1(c)] had been largely lost during leaf senescence.

Partial purification of PR proteins from healthy and infected plants

Figure 2(a) and (b) shows non-denaturing gels of partially purified PR proteins from leaves of young, infected (I) and old, healthy (H) plants. The fraction eluted from DEAE cellulose at 200 mM NaCl contained only one major band, corresponding in mobility to PR1a [Fig. 2(a)]. The fraction eluting at 150 mM NaCl had a more complex pattern, but was highly enriched for PR1b [Fig. 2(b)]. Antoniw *et al.* [2] achieved a further purification of PR1b, by using 3 chromatographic steps rather than the 2 used for Fig. 2(b).

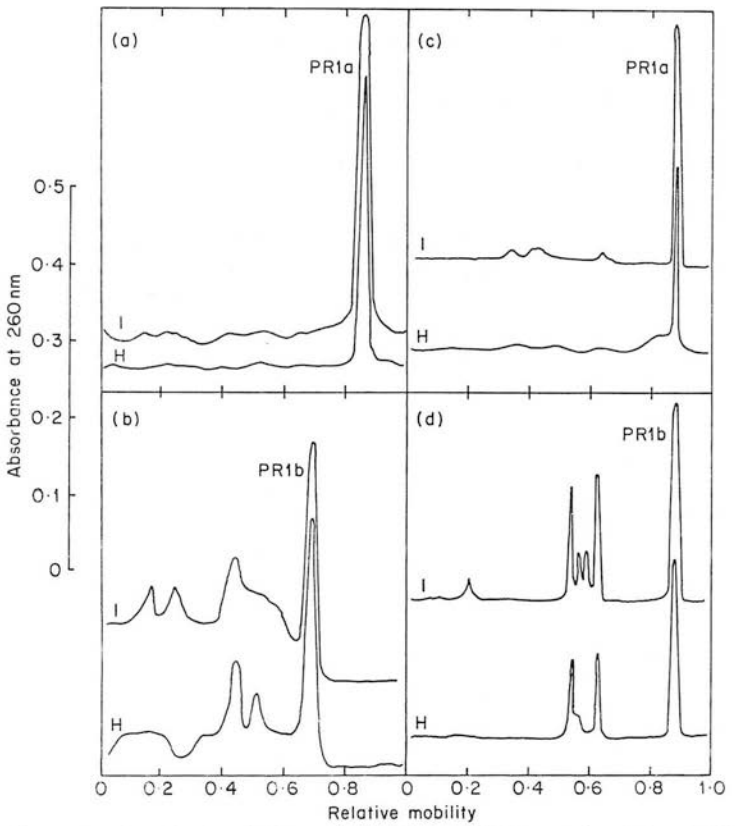

FIG. 2. Electrophoresis of partially purified PR1a and PR1b proteins. (a) and (c) Proteins eluted from DEAE cellulose at 200 mM NaCl. (b) and (d) Proteins eluted at 150 mM NaCl. (a) and (b) show fractionations on non-denaturing gels; (c) and (d) fractionations on denaturing (SDS) gels. Gel scans marked I are from leaves of young plants with an average of 50 lesions per leaf, extracted 6 days after inoculation with TMV. Scans marked H are from leaves 450 to 700 mm high on healthy, untreated plants which were in flower at the time of sampling.

On denaturing (SDS) gels, the highly purified PR1a fractions from young infected or old, healthy leaves showed a single band of high mobility [Fig. 2(c)]. Its molecular weight, measured by co-electrophoresis of marker proteins (Dalton Mark VI molecular weight markers, Sigma Chemical Co.) was 14 000. This agrees with the results of Antoniw *et al.* [2] for protein extracted from infected leaves. The PR1b

fractions also showed a prominent band of this mobility, together with some minor peaks [Fig. 2(d)]. This result supports the suggestion by Antoniw *et al.* [*2*] that PR1b is a charge isomer of PR1a, with the same molecular weight.

Partially purified PR1a and PR1b fractions from old healthy plants, were mixed with the corresponding partially purified fractions from young, infected plants, then fractionated by electrophoresis on denaturing and non-denaturing gels. The PR1a and PR1b proteins were again detected as single, symmetrical bands. This provides confirmatory evidence that the proteins from the two sources had identical electrophoretic mobilities.

Thus the PR1a and PR1b proteins from young infected and from old healthy leaves co-purified during chromatography, and behaved identically when fractionated on 2 different gel systems. Proteins extracted from young healthy leaves and partially purified by column chromatography showed no traces of PR1a or PR1b bands on either type of gel.

Effects of decapitation and removal of lower leaves

Table 2 shows that if either the developing inflorescence or the lower leaves, or both, were removed from 900 mm-tall plants, the amount of PR1a protein found 14 days later was very much less than in control plants, which had flowered and on which the lower leaves had yellowed during this period. Accumulation of the other PR proteins was similarly prevented by decapitation or removal of the lower leaves.

TABLE 2

Numbers and diameters of lesions formed on leaves of mature Xanthi-nc. tobacco plants, and concentration of the pathogenesis-related protein PR1a[a]

Treatment	Lesions per half leaf[b]	Lesion diameter (mm)[b]	PR1a concentration (relative units g^{-1})
A Control	8·4±1·5[c]	1·25±0·11[d]	20·4
B Lower leaves removed	13·4±1·8	1·21±0·10	1·8
C Inflorescence removed	7·6±2·5	1·60±0·10	3·9
D Lower leaves and inflorescence removed	12·4±1·5	1·77±0·15	1·1

[a] The developing inflorescence, and/or lower leaves showing any signs of yellowing, were removed from 900 mm tall plants. Fourteen days later, PR proteins were extracted from leaves situated between 450 and 700 mm high on the plants. Similar leaves were inoculated with TMV at 5 μg ml^{-1}.

[b] Values are means±standard errors.

[c] Using Kleczkowski's transformation [*10*] for lesion numbers with low means, the treatments are not significantly different at $P = 0{\cdot}05$. Similarly transformed, lesion number on all plants with lower leaves removed (treatments B+D) is significantly higher than on all plants with lower leaves remaining (treatments A+C) at $P = 0{\cdot}01$.

[d] Least significant difference between treatments at $P = 0{\cdot}05$ is 0·33 mm.

When these plants were inoculated with TMV, at 14 days after treatment, no correlation was found between concentration of PR1a proteins, and either the size or number of lesions formed. For example, control plants contained more than 10 times as much PR1a as plants from which the lower leaves had been removed, but formed lesions of the same size. Control plants contained more than 5 times as much PR1a as decapitated plants, but formed similar numbers of lesions.

Two influences were, however, apparent: removal of the inflorescence in any treatment led to a significant increase in lesion diameter, and removal of the lower leaves led to a significant increase in lesion number.

Finally, it should be noted that the numbers of lesions formed on any of the old plant treatments were considerably lower (around one-tenth) than the numbers caused by the same inoculum on leaves of young plants. Lesions on old and young plants were, however, of similar sizes.

DISCUSSION

This paper confirms previous reports that PR proteins are not detectable in young, healthy plants [*2*, *8*, *14*, *16*]. A report by Barker [*3*], suggesting that PR proteins were present in young, healthy plants was challenged by van Loon [*14*], who suggested that Barker's conclusion was based on a misinterpretation of protein band patterns on gel fractionations.

The results in this paper suggest that the 4 PR proteins do accumulate in healthy, untreated plants as they begin to flower and senesce. However, co-migration on a single gel system is not by itself enough to prove identity of proteins extracted from different sources. The healthy plant PR1a and PR1b proteins were therefore partially purified and shown to behave identically to the infected plant PR1a and PR1b proteins by the following criteria: they eluted from gel filtration columns at the same volume, and from ion-exchange chromatography at the same salt concentrations, and had the same mobilities when fractionated by electrophoresis under both denaturing and non-denaturing conditions. There can therefore be little doubt that the PR1a and PR1b proteins from healthy, mature plants are the same as those from young infected plants.

The amount of PR protein accumulated in healthy, mature plants equalled or exceeded the concentrations found in uninoculated parts of young, TMV-infected plants, during the period when these parts displayed acquired systemic resistance.

The induction of PR proteins in mature plants appeared to depend on the presence of both the senescing lower leaves and the developing inflorescence: removal of either caused a major inhibition of PR protein accumulation. A possible explanation is that a signal coming from one end of the shoot, as a result of developmental changes, elicits a second signal from the other end. This second signal then triggers the PR protein accumulation found in all leaves of the plant. Alternatively, the PR proteins might actually be synthesized in either apex or lower leaves, and transported throughout the plant.

The occurrence of PR proteins in healthy plants brings their role in any induced, antiviral defence mechanism into question. It seems unlikely that they are involved in any specific response to the pathogen analogous to the mammalian antibody or interferon systems.

It might be argued that the comparatively low numbers of lesions produced on leaves of old plants is due in part to their content of PR proteins. However, leaves of old plants are also thick and leathery, and may simply be more difficult to inoculate mechanically than the thinner, more fragile leaves of young plants. The lack of correlation between the very different amounts of PR proteins found in mature plants from which different parts had been removed, and the numbers and sizes of lesions

formed after inoculation, is also difficult to reconcile with a role of these proteins in acquired systemic resistance.

I thank Su Loughlin for capable technical assistance.

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Printed in Great Britain by Henry Ling Ltd., at the Dorset Press, Dorchester, Dorset

J. gen Virol. (1982), **58**, 305–313. *Printed in Great Britain*

Key words: *TMV/systemic resistance/pathogenesis-related proteins*

Are 'Pathogenesis-related' Proteins Involved in Acquired Systemic Resistance of Tobacco Plants to Tobacco Mosaic Virus?

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(*Accepted 5 October 1981*)

SUMMARY

Four host-coded 'pathogenesis-related' proteins accumulate systemically in local-lesion-forming varieties of tobacco after infection with tobacco mosaic virus. It has been suggested that they are involved in the acquired systemic resistance of plants to a second inoculation. Pathogenesis-related protein concentration and amount of resistance (reduction in size and number of lesions formed in the second inoculation) were measured at various times after the first inoculation. The results showed no quantitative or temporal relationship between amounts of resistance and pathogenesis-related proteins. In particular, resistance could be demonstrated in leaves before detectable accumulation of pathogenesis-related protein. Abscisic acid sprayed on plants induced an apparent resistance without inducing pathogenesis-related proteins. Low doses of methyl benzimidazol-2yl-carbamate caused accumulation of pathogenesis-related protein but not resistance. *Nicotiana glutinosa* plants accumulated large amounts of a similar protein after infection, but became more susceptible to a second inoculation. All these results suggest that the pathogenesis-related proteins do not play a central role in the mechanism of acquired systemic resistance.

INTRODUCTION

When tobacco cultivars containing the *N* gene are inoculated with tobacco mosaic virus (TMV), the virus is restricted to the local lesions which form around the infection sites (Holmes, 1938). If half of a lower leaf is inoculated, the uninoculated half and upper leaves become apparently resistant to a subsequent challenge inoculation. This 'induced' or 'acquired systemic resistance' is expressed as a reduction in size and/or number of lesions formed after challenge inoculation (Ross, 1961, 1966; Kassanis *et al.*, 1974; van Loon, 1975).

At least four new host-coded proteins are detected systemically after the first inoculation (Gianinazzi *et al.*, 1970; van Loon & van Kammen, 1970; Kassanis *et al.*, 1974). They are generally absent from healthy plants (van Loon, 1976) and have been referred to as 'pathogenesis-related' proteins (Antoniw *et al.*, 1980). It has been suggested that they may be involved in acquired systemic resistance (van Loon & van Kammen, 1970; van Loon, 1975; Kassanis *et al.*, 1974; Kassanis & White, 1974). However, attempts to demonstrate a direct antiviral effect do not seem to have been successful (Kassanis *et al.*, 1974; Kassanis & White, 1978).

In this paper I report experiments which tested the involvement of the pathogenesis-related proteins in acquired systemic resistance.

0022-1317/82/0000-4743 $02.00

METHODS

Plants and viruses. Nicotiana tabacum L. cv. Xanthi-nc and *N. glutinosa* L. were grown in 12·5 cm diam. pots of Levington compost. Plants which were treated with methyl benzimidazol-2yl-carbamate (MBC) or their untreated controls were grown in John Innes no. 2 compost. All plants were kept under natural light in a glasshouse, with a minimum temperature of 16 °C and a maximum of 20 to 25 °C.

TMV tomato strain O was multiplied in *N. tabacum* cv. Samsun and purified using the method of Gooding & Hebert (1967). Expanded leaves on 25 cm-tall plants of cv. Xanthi-nc were inoculated by rubbing with a suspension of TMV at 1 to 5 μg/ml 50 mM-sodium phosphate buffer pH 7. Carborundum was used as abrasive; leaves were washed with water after inoculation. There were 10 to 12 leaves per treatment, on 5 or 6 plants. Seven days after inoculation, lesions were counted and the diam. of 60 to 100 lesions per treatment measured using a stereoscopic microscope with ×20 magnification. Differences in lesion size or number between treatments were tested for significance by *t*-test; lesion numbers were first log-transformed using Kleczkowski's (1949, 1955) transformations.

For measurement of acquired systemic resistance, plants of cv. Xanthi-nc or *N. glutinosa* were inoculated on one-half of each of two lower leaves (the primary inoculation). Control plants were sham-inoculated with buffer and carborundum. At various times afterwards, plants were challenge-inoculated on two upper leaves, and on the previously uninoculated halves of the two lower leaves. Acquired systemic resistance was measured as the percentage reduction in lesion size or number caused by the primary inoculation with TMV.

Protein extraction and electrophoresis. For each treatment, leaves were harvested from at least five plants. Samples of 5 g were ground at 0 °C in 5 ml pH 2·8 buffer (84 mM-citric acid; 32 mM-Na_2HPO_4; 14 mM-2-mercaptoethanol) (Antoniw *et al.*, 1980). The homogenate was filtered through muslin and centrifuged at 10000 ***g*** for 20 min. A 5% amount of sucrose (w/v) and a trace of bromophenol blue were added to the supernatant. Samples of 10 to 500 μl of supernatant were fractionated by electrophoresis on 10% polyacrylamide gels (0·67% cross-linked), 75 mm long × 6 mm diam., for 5 h at 3 mA per gel constant current. Buffers were as described by Davis (1964). After electrophoresis, gels were stained overnight in 0·1% PAGE blue G-90 (BDH) in 40% methanol: 10% acetic acid, then destained in 14% methanol: 10% acetic acid. Destained gels were stored in 7% acetic acid. Mobilities of protein bands (R_F) were expressed relative to that of the bromophenol blue marker. Gels were scanned for absorbance at 260 nm in a Joyce Loebl Gel Scanner; PAGE blue G-90 has an absorption maximum at this wavelength. The peak area of a protein band was shown to be proportional to the weight of protein in the band by electrophoresis of different amounts of the same sample. However, intensity of staining varied from experiment to experiment. To allow comparisons between experiments, samples of a standard extract were fractionated with each batch of gels.

RESULTS

Pathogenesis-related proteins

Fig. 1 (*a*) shows electrophoresis of proteins from TMV-infected leaves of cv. Xanthi-nc, with an average of 50 lesions per leaf, extracted 6 days after inoculation. The scan shows four highly mobile bands which are not visible in proteins extracted from comparable, sham-inoculated healthy leaves (Fig. 1 *b*). The four bands from infected leaves are designated PR1a, PR1b, PR1c and PR2 in order of decreasing mobility, and using the nomenclature system proposed by Antoniw *et al.* (1980). The R_F values of the bands in 10% gels were 0·86, 0·69, 0·59 and 0·53 respectively. These values correspond well with those reported by Antoniw *et al.* (1980). PR1a was by far the most prevalent band.

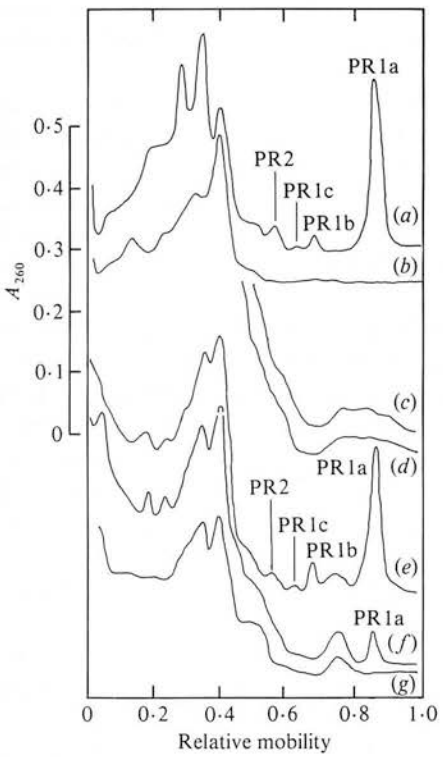

Fig. 1

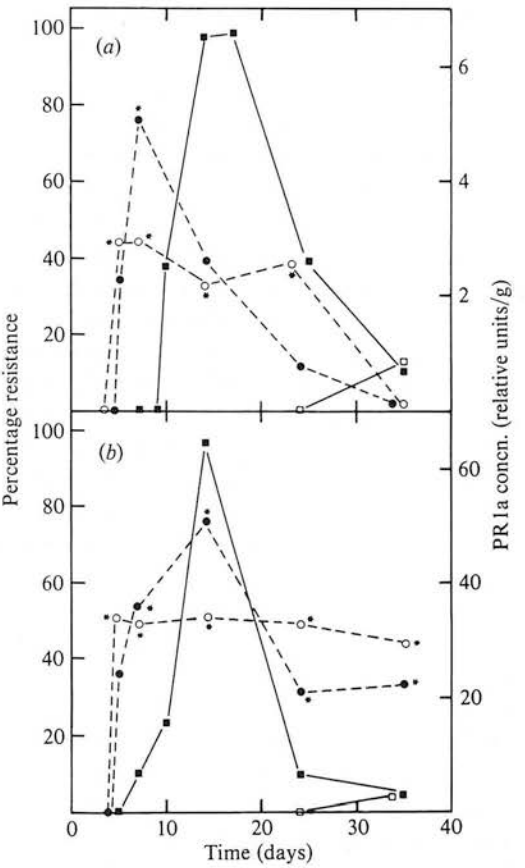

Fig. 2

Fig. 1. Polyacrylamide gel electrophoresis of proteins extracted from healthy and TMV-infected plants of *N. tabacum* cv. Xanthi-nc. (*a*) From leaves bearing an average of 50 lesions, 6 days after inoculation; (*b*) from comparable sham-inoculated healthy leaves; (*c*) from healthy leaves on plants sprayed for 14 days with 0·5 mM-ABA; (*d*) from comparable leaves on control plants sprayed with water; (*e*, *f*) from young expanding leaves on healthy plants treated respectively with 2 g or 20 mg MBC; (*g*) from comparable leaves on untreated control plants. The gels were loaded with protein equivalent to the following fresh wt. of leaf: (*a*, *b*) 50 mg; (*c*, *d*) 250 mg; (*e*, *f*, *g*) 100 mg.

Fig. 2. Changes in acquired systemic resistance and concentration of PR1a protein with time after inoculation with TMV. At day 0, *N. tabacum* cv. Xanthi-nc plants were inoculated on one-half of each of two lower leaves; these formed 50 to 100 lesions per half-leaf. Control plants were sham-inoculated. The concentration of PR1a protein in infected (■——■) or control (□——□) plants was measured in upper leaves (*a*) and in the uninoculated half of lower leaves (*b*). Acquired systemic resistance was measured in upper and lower half-leaves by challenge inoculation at various times and expressed as the percentage reduction in lesion number (●---●) or diameter (○---○) caused by the primary inoculation. For points marked (*) the difference between control and primary inoculated treatments was significant at $P = 0{\cdot}05$.

The infected leaf protein extract shown in Fig. 1 (*a*) was used as the standard for calibration of staining intensity in each batch of gels. The concentration of PR1a protein in the standard was defined as 100 units/g fresh wt. Amounts of PR1a protein in other extracts were expressed relative to this. The limit of detection was less than 0·1 unit/g. The four PR proteins were also detected in parts of infected plants which had not been inoculated and which did not develop lesions.

Time courses of accumulation of pathogenesis-related proteins and development of acquired systemic resistance

Fig. 2 shows changes in concentration of PR1a in uninoculated parts of the plant with time after inoculation of one-half of each of two lower leaves. Similar changes occurred in PR1b and PR2 concentration. PR1c appeared to show similar changes, although the amount was generally too low for accurate quantification.

In the uninoculated halves of lower inoculated leaves, PR1a became detectable 7 days after the primary inoculation. In uninoculated upper leaves PR1a was first detected 10 days after inoculation of lower leaves. In both cases, the concentration rose to a maximum around 14 to 17 days after inoculation then declined. This interpretation makes the assumption that PR proteins are extracted with the same efficiency as leaves age, an assumption justified by the large amounts of PR proteins recoverable from leaves of senescent, flowering healthy plants (Fraser, 1981).

Upper leaves, and uninoculated halves of lower leaves on both TMV-infected and sham-inoculated control plants were challenge-inoculated with TMV at various times after the first inoculation. The data for percentage acquired resistance are plotted in Fig. 2 at the time of challenge inoculation. Actual lesion sizes and numbers were recorded 7 days later, but differences in lesion size and number between control and 'resistant' plants were clearly established by 3 days after challenge inoculation. The PR protein concentration in the 3 days after challenge inoculation is, therefore, most relevant when investigating the correlation between resistance and PR protein concentration. Control experiments also showed that differences in lesion size and number between treatments were maintained subsequent to the 7th day after challenge inoculation.

Comparing upper leaves and lower opposite half-leaves, it was clear that the amount of resistance induced was generally similar. From 5 to 24 days after primary inoculation, resistance measured as reduction in lesion size was about 40% in upper leaves and 50% in lower leaves. But during this period, the concentration of PR1a in lower half-leaves was around 10 times that in upper leaves.

Within each type of leaf, there was no clear correlation between amount of resistance (measured as reduction in lesion size) and amount of PR protein at various times after primary inoculation. In particular, in lower half-leaves, the resistance remained constant from 5 to 35 days, but PR1a protein concentration varied over a range of more than 30-fold.

In both types of leaf, the curve for resistance (measured as reduction in lesion number) was more similar in shape to the curve for PR1a protein concentration than was the curve for resistance based on lesion size. However, the changes in resistance measured as lesion number tended to develop in advance of the matching changes in PR1a protein concentration, especially in upper leaves. In upper leaves, resistance was detectable when leaves were challenge-inoculated 5 days after primary inoculation; differences in the size and numbers of lesions formed were clearly established by day 9. However, no PR1a protein (or other PR protein) could be detected in this experiment until 10 days after the primary inoculation.

As healthy plants age and begin to flower, they begin to accumulate PR proteins (Fraser, 1981). By 35 days, lower half-leaves on sham-inoculated plants contained similar concentrations of PR1a to comparable leaves on TMV-infected plants (Fig. 2*b*). However, the lower half-leaves on infected plants still showed significant resistance, measured on a basis of either lesion size or number.

Effects of treatment with abscisic acid

Healthy Xanthi-nc plants were sprayed daily with 0·5 mM-*cis-trans*-abscisic acid (ABA) for 14 days. Control plants were sprayed with water. After 14 days, proteins were extracted from expanded leaves and similar leaves were inoculated with TMV. The scans (Fig. 1*c*

Table 1. *No. and diam. of local lesions formed on plants inoculated with TMV after various pretreatments, and concentration of PR1a protein at the time of inoculation**

Species	Pretreatment	Lesion diam. (mm)	Lesions/leaf or half-leaf	PR1a concn. (relative units/g)
N. tabacum cv. Xanthi-nc	Control	2·19 ± 0·07	221 ± 41	
	Abscisic acid	1·91 ± 0·07†	66 ± 8†	
N. tabacum cv. Xanthi-nc	Control	2·04 ± 0·05	23 ± 6	ND‡
	MBC (2 mg)	2·01 ± 0·08	25 ± 6	ND
	MBC (20 mg)	1·96 ± 0·06	21 ± 7	4·9
	MBC (200 mg)	2·12 ± 0·06	28 ± 5	12·2
	MBC (2 g)	1·03 ± 0·04†	4 ± 2†	45·3
N. glutinosa	Control	1·27 ± 0·06	60 ± 9	
	Pre-inoculated	1·35 ± 0·05	95 ± 12	

* *N. tabacum* plants were given various doses of MBC 9 and 2 days before inoculation, or were sprayed with 0·5 mM-ABA daily for 14 days before inoculation. *N. glutinosa* plants were inoculated on one-half of two lower leaves; controls were sham-inoculated. Seven days later the opposite halves were challenge-inoculated. All values are means ± standard errors.

† Significantly different from the control at $P = 0{\cdot}05$.

‡ ND, Not detectable.

and *d*) show that ABA treatment did not induce any detectable PR proteins: no bands were seen even in gels very heavily loaded with protein. However, ABA did cause a 70% reduction in lesion number and a small, though statistically significant reduction in lesion size (Table 1). The reduction in lesion size and number, which was found consistently in replicate experiments, contrasts with previous reports of an increase in lesion number or size after ABA treatment at the same concentration (Balazs *et al.*, 1973; Bailiss *et al.*, 1977). The explanation for this apparent discrepancy lies in the different methods used to apply ABA. Balazs *et al.* (1973) and Bailiss *et al.* (1977) infiltrated leaf discs with ABA solution. This leads to much larger increases in internal ABA concentration than spraying leaves on intact plants. For example, treatment of leaf discs by infiltration with 50 μM-ABA for 24 h raised internal ABA concentration by around 10 μg/g (Whenham & Fraser, 1980), whereas daily spraying for 8 days with the same concentration raised internal ABA concentration by only 60 ng/g (Whenham & Fraser, 1981*a*).

Balazs *et al.* (1973) and Bailiss *et al.* (1977) reported visible damage resembling senescence at ABA concentrations which caused increased lesion number and size; the internal ABA concentrations of their leaf discs were undoubtedly far beyond the normal physiological range. In contrast, the ABA-sprayed plants used in this study showed no signs of damage or enhanced senescence, but were slightly darker green than control plants. The increase in their internal ABA concentration was calculated to be around 600 ng/g fresh wt. (Whenham & Fraser, 1981*a*) which is within the range of normal physiological concentrations found in tobacco leaves (Whenham & Fraser, 1981*b*).

Effects of treatment with MBC

Xanthi-nc plants were treated with various doses of MBC by watering the pots with a suspension of 'Bavistin' (BASF Agrochemicals; 50% MBC by wt.). In each treatment, plants received two equal doses at day 0 and day 7. At day 9 proteins were extracted and fractionated. Plants treated with a total of 2 mg MBC did not develop detectable PR protein (Table 1). Plants receiving 20 or 200 mg MBC showed detectable amounts of PR1a (Fig 1*f*, Table 1). The actual amounts of PR1a induced were comparable to those found in upper leaves of TMV-infected plants showing acquired systemic resistance (Fig. 2*a*). The broad peak of protein with a mobility of 0·75 present in both MBC-treated and control leaves

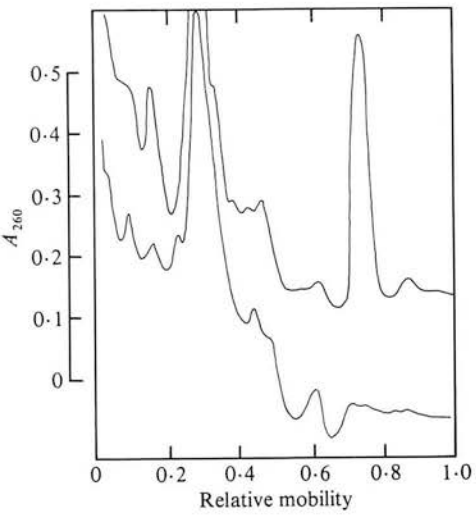

Fig. 3. Polyacrylamide gel electrophoresis of proteins extracted from healthy and TMV-infected *N. glutinosa* plants. (*a*) Extracted from uninoculated halves of leaves 7 days after inoculation of the opposite halves; (*b*) from comparable leaves on sham-inoculated control plants. Gels were loaded with protein equivalent to 100 mg fresh wt. of leaf.

(Fig. 1*f*, *g*) was a host protein characteristic of younger leaves; it disappeared as the leaf completed expansion (Fig. 1 *b*).

The PR1a protein induced by 200 mg MBC was partially purified by gel-filtration and ion-exchange chromatography (Antoniw *et al.*, 1980) as described earlier (Fraser, 1981) and was found to co-migrate with authentic, TMV-induced PR1a protein when fractionated by electrophoresis in non-denaturing and denaturing (SDS) gels (data not shown). Thus, there can be little doubt that the protein induced by MBC was the same as the PR1a induced by TMV.

MBC doses of 20 or 200 mg/plant did not induce detectable amounts of the three minor PR proteins 1b, 1c and 2. Plants treated with 2 g MBC had high concentrations of PR1a and also detectable PR1b, 1c and 2 (Table 1, Fig. 1*e*). Nine days after the first MBC treatment, comparable leaves on further plants were inoculated with TMV; lesion size and number were subsequently measured. Treatment with MBC at up to 200 mg per plant caused no significant change in size or number of lesions, although plants treated with 20 or 200 mg MBC did contain detectable PR1a protein (Table 1). Plants treated with 2 g MBC formed significantly fewer and smaller lesions than the controls. However, 2 g MBC also caused direct phytotoxic effects by 9 days, including reduced growth, yellowing and necrotic spots on uninfected plants. The effect on lesion development may, therefore, have been a result of this phytotoxicity.

A pathogenesis-related protein in N. glutinosa

Leaves on *N. glutinosa* plants were inoculated on one-half with TMV and formed an average of 50 lesions. Seven days later, proteins were extracted from the uninoculated halves. Comparable uninoculated halves were challenge-inoculated. Fig. 3 shows that TMV infection caused accumulation of very large amounts of a protein with mobility 0·74 in the uninoculated half of the leaf. Van Loon & van Kammen (1970) also found a band of similar mobility. Fig. 3 shows that the *N. glutinosa* protein is similar to the PR proteins of *N. tabacum* in that it is highly soluble at pH 2·8.

Table 1 shows that half-leaves opposite previously inoculated halves appeared to form more lesions than the controls when challenge-inoculated. In replicate experiments, the primary inoculated plants formed 1·2 to 1·7 times as many lesions as the controls. There was no difference in lesion size. Upper leaves of TMV-infected *N. glutinosa* plants also contained the PR-like protein and formed significantly more lesions after challenge-inoculation than control plants; lesion size on upper leaves was unaltered or very slightly reduced as a result of prior inoculation of lower leaves (Fraser *et al.*, 1979).

DISCUSSION

The four PR proteins have been shown to occur in leaves which also show acquired resistance as a consequence of localized TMV infection elsewhere in the plant (Kassanis *et al.*, 1974; van Loon, 1975; Rohloff & Lerch, 1977). Several experimental treatments applied to healthy plants will also induce PR proteins and resistance to TMV. These include polyacrylic acid (Gianinazzi & Kassanis, 1974), acetylsalicylic acid (White, 1979) and plant hormones (Antoniw *et al.*, 1981). Thus, the evidence for involvement of PR proteins in acquired systemic resistance is comprehensive but entirely correlative.

In this paper, PR protein concentration and amount of resistance were measured at various times after the primary inoculation, to test further the correlation between the proteins and resistance. The results suggest that there is not a close quantitative relationship between the amount of PR protein present and the amount of acquired systemic resistance. Furthermore, examination of the temporal relationship between changes in PR protein concentration and amounts of resistance also provided evidence against a role of the proteins in resistance. In particular, resistance could be demonstrated in leaves before detectable accumulation of PR protein occurred.

The second experimental approach was to attempt to induce PR proteins and resistance independently of each other. ABA induced an apparent resistance, measured as a reduction in lesion number, without inducing detectable PR proteins. ABA had, however, only a small effect on lesion size. Treatment with low doses of MBC induced accumulation of significant amounts of PR1a protein without inducing any resistance, although higher, phytotoxic doses of MBC induced both proteins and apparent resistance. Finally, *N. glutinosa* was shown to accumulate large amounts of a protein similar to the *N. tabacum* PR protein after TMV infection. But in *N. glutinosa*, this protein was associated with an increase in susceptibility to infection and not with resistance. Thus, these experiments indicated that it was possible to induce PR protein without resistance and that effects similar to resistance could be induced without induction of PR protein.

A necessary qualification to the experiments with chemical induction of PR proteins or apparent resistance is that the mechanisms involved might not be the same as with virus as inducer. This does not, however, exclude them as reasonable experimental tests. Are the PR proteins therefore involved in acquired systemic resistance? Logically, it is impossible to prove the negative statement that PR proteins are not involved. Any model relating proteins to resistance can theoretically be modified to accommodate additional experimental evidence. However, the different types of experimental test described in this paper all suggest that the PR proteins are not centrally involved in acquired systemic resistance. This suggestion raises two further questions: what is the function of PR proteins if not resistance, and, what is the mechanism of the apparent systemic resistance? The wide variety of factors which will induce PR proteins: viral infection (Gianinazzi *et al.*, 1970; van Loon & van Kammen, 1970); fungal infection (Gianinazzi *et al.*, 1980); various chemical treatments (Gianinazzi & Kassanis, 1974; White, 1979; Antoniw *et al.*, 1980) and flowering of the plant (Fraser, 1981) suggests that the PR proteins are some general response to stress conditions. The two aspects of acquired systemic resistance, reduction in lesion size and

number, seem to involve separate mechanisms, as they can apparently operate to some extent independently (e.g. Fig. 2, Table 1 and Fraser *et al.*, 1979).

Alteration in lesion number does show an inverse correlation with changes in ABA concentration. In *N. glutinosa*, which shows an increase in lesion number as a result of the first inoculation, ABA concentration in the challenge inoculated leaves is reduced (Fraser *et al.*, 1979). In *N. tabacum*, where lesion number is reduced as a result of the first inoculation, ABA concentration of the challenge-inoculated leaves is increased by the primary inoculation (Whenham & Fraser, 1981 *a*). The reduction in number of lesions formed when healthy plants were sprayed with ABA before inoculation is also consistent with this correlation. Whether ABA is the direct cause of altered lesion formation, or indirectly involved through an effect on leaf water status and mechanical susceptibility to inoculation (Cassells *et al.*, 1978) remains to be seen.

Reduction in lesion size could involve actual inhibition of virus multiplication, or merely a retardation of necrotization. Evidence that virus multiplication is reduced in acquired systemic resistance is conflicting (Ross, 1966; van Loon & Dijkstra, 1976; Balazs *et al.*, 1977; Fraser, 1979) and the topic requires further study.

I thank Su Loughlin for capable assistance.

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(Received 18 May 1981)

Reprinted from *Physiological Plant Pathology* (1982) **20,** 109–117

Effects of temperature on the *Tm-1* gene for resistance to tobacco mosaic virus in tomato

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(Accepted for publication October 1981)

In tomato plants grown at constant temperatures from 20 to 35 °C, the *Tm-1* resistance gene was completely effective in suppressing symptom formation by tobacco mosaic virus (TMV) strain 0. In contrast, inhibition of virus multiplication by the gene was strongly temperature-dependent; in plants heterozygous for *Tm-1* it was over 95% at 20 °C but only 20% at 33 °C. Although TMV multiplication in both susceptible and resistant plants was reduced at very high growth temperatures, it is suggested that this general effect of temperature is distinct from its specific effect on *Tm-1* controlled inhibition of virus multiplication.

TMV strain 1 caused symptoms on *Tm-1* plants; the higher the temperature, the more severe the symptoms. Strain 1 multiplication in both susceptible and resistant plants was much more temperature sensitive than was strain 0 multiplication and this tended to obscure the effects of temperature on the action of the *Tm-1* gene with respect to strain 1 multiplication. However, the results indicate that strain 1 multiplication is inhibited by *Tm-1* at 25 °C but not at 33 °C.

Experiments in which *Tm-1* plants infected with strain 0 were transferred from high to low temperature suggested that the anti-multiplication activity of *Tm-1* was quickly restored at the lower temperature. Under this temperature regime, strain 0 did overcome the symptom suppression function of the gene.

INTRODUCTION

In tomatoes, the *Tm-1* gene confers resistance to common (strain 0) isolates of tobacco mosaic virus (TMV) [*3, 12, 13*]. Under normal glasshouse conditions, the formation of mosaic symptoms is completely suppressed in both heterozygous (*Tm-1*/+) and homozygous (*Tm-1*/*Tm-1*) hosts. TMV multiplication is inhibited by about 70% in *Tm-1*/+ plants and between 90 and 95% in *Tm-1*/*Tm-1* plants [*5*]. TMV strain 1 [*13*] causes symptoms on plants containing the *Tm-1* gene, but under glasshouse conditions does not completely overcome the inhibitory effects of the gene on multiplication [*6*].

Temperature has been reported to influence the effects of several genes for resistance to TMV. In tobacco, the *N* gene causes restriction of virus to necrotic local lesions at normal temperatures, but permits systemic spread at 35 °C [*11*]. Tomato plants with the *Tm-2* or *Tm-2*^{2} genes for TMV resistance are either completely symptomless, or display only slight local necrosis, after inoculation with strain 0 at normal temperatures. At elevated temperatures, severe systemic necrotic reactions have been reported [*1, 13–15*]. Most studies of the effects of temperature on resistance gene action have concentrated on symptoms while effects on virus multiplication have received little or no attention.

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The temperature sensitivity of the activity of the *Tm-1* gene has not been studied in any detail. In this paper we report an investigation of the effects of temperature on its two functions, symptom suppression and inhibition of virus multiplication.

MATERIALS AND METHODS

Plants and viruses

Near-isogenic lines of tomato (*Lycopersicon esculentum* Mill. cv. Craigella) were obtained from Dr T. J. Hall, Glasshouse Crops Research Institute, Littlehampton, Sussex, U.K. Line GCR 26 is susceptible (+/+); J 484 heterozygous and GCR 237 homozygous for *Tm-1*. Plants were grown in Levington Universal compost in 12·5 cm diameter pots, in a glasshouse with a 15 °C night and 20 to 25 °C day temperature regime.

For controlled temperature experiments, plants were grown in Fisons controlled environment cabinets at constant temperatures between 20 and 35 °C. Daylength was 14 h, and irradiance 50 W m^{-2}.

TMV tomato strains 0 and 1 (GCRI isolates; [*13*]) were multiplied in tobacco (*Nicotiana tabacum* L. cv. Samsun) and purified by polyethylene glycol precipitations [*8*]. Tomato plants 10 to 20 cm tall were inoculated on two expanded leaves by rubbing with a suspension of 10 μg TMV ml^{-1} 50 mM sodium phosphate buffer, pH 7. Carborundum (400-mesh) was used as an abrasive and leaves were washed with running water immediately after inoculation.

Symptom scoring

To eliminate subjective bias, plants were scored "blind" for symptom severity, i.e. without the observer knowing host genotype or treatment. Symptom severity was expressed on the scale:

0 symptom free
1 trace of systemic mosaic
2 well developed mosaic, with mottling of young leaves
3 severe mosaic with stunting and distortion of upper parts
4 as 3, but with small areas of leaf necrosis (normally less than 10% of total leaf area)
5 extensive or complete necrosis; plants dead.

Grade 3 symptoms were typically the most severe found under glasshouse conditions.

Measurement of TMV RNA

At each sampling time, leaflets were taken from the inoculated leaves, and separately from upper leaves which had become infected by systemic spread of virus. Generally at least 4 to 6 plants were sampled for each treatment. Midribs were removed and the laminas roughly chopped and mixed. Nucleic acids were extracted from 3 or more replicate subsamples for each treatment [*7*] and fractionated by electrophoresis on 2·1% polyacrylamide gels [*10*] at 8 V cm^{-1}; 5 mA gel^{-1} for 4 h. TMV RNA concentration per g fresh wt was calculated from the peak area of the TMV RNA on the ultraviolet absorption scan of each gel [*4*].

RESULTS

Effects of Tm-1 *on TMV strain 0 at different temperatures*

In susceptible plants, the severity of mosaic symptoms produced by TMV strain 0 increased with temperature [Fig. 1(a)]. At 35 °C, many plants were killed by the virus within 2 weeks of inoculation. At 33 °C, most plants developed slight necrosis within 2 or 3 weeks of inoculation; some were ultimately killed. Inoculated plants heterozygous or homozygous for the *Tm-1* gene did not develop mosaic symptoms at any temperature tested. Visually, infected *Tm-1* plants resembled healthy plants grown at the same temperature.

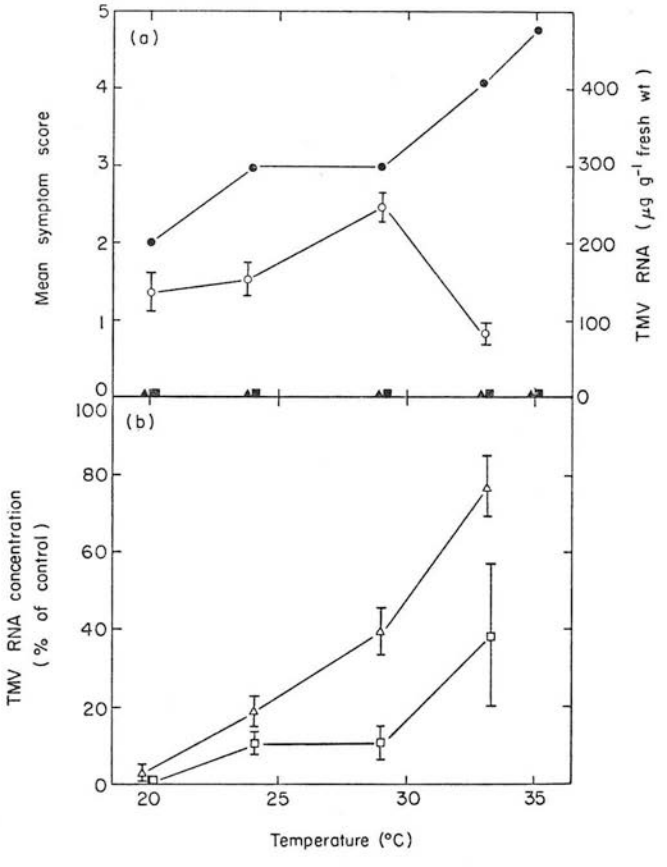

FIG. 1. Symptom severity and TMV RNA accumulation in tomato plants of different resistance genotypes, grown at different temperatures, and inoculated with TMV strain 0. Plants were grown at the indicated temperatures for 8 days before, and after inoculation. Symptoms were assessed and nucleic acids extracted 19 days after inoculation. (a) Mean symptom scores in (●) +/+, (▲) *Tm-1*/+ and (■) *Tm-1*/*Tm-1* plants. (○) TMV RNA concentration in systemically-infected leaves of +/+ plants. Values are means ± standard errors of 4 replicate determinations. (b) TMV RNA accumulation in systemically-infected leaves of (△) *Tm-1*/+ and (□) *Tm-1*/*Tm-1* plants. Results are expressed as a percentage of the TMV RNA accumulation in comparable leaves of +/+ plants grown at the same temperature.

TMV multiplication was followed by measuring the accumulation of TMV RNA. The accumulation of strain 0 RNA in +/+ plants was temperature dependent [Fig. 1(a)]. It increased with temperature from 20 to 29 °C, then was lower at 33 °C. Similar effects were found in directly inoculated and systemically infected leaves. Figure 2 shows time courses of symptom development and RNA accumulation in +/+ plants grown at 29 and 33 °C. At 33 °C, small areas of leaf necrosis were first visible (symptom score 4) 15 to 20 days after inoculation. However, the rate of RNA accumulation at 33 °C was lower than at 29 °C right from the time of inoculation. We conclude that the lower accumulation of RNA at high temperature in +/+ plants was therefore a direct inhibitory effect of temperature on TMV multiplication, and not an indirect consequence of necrosis.

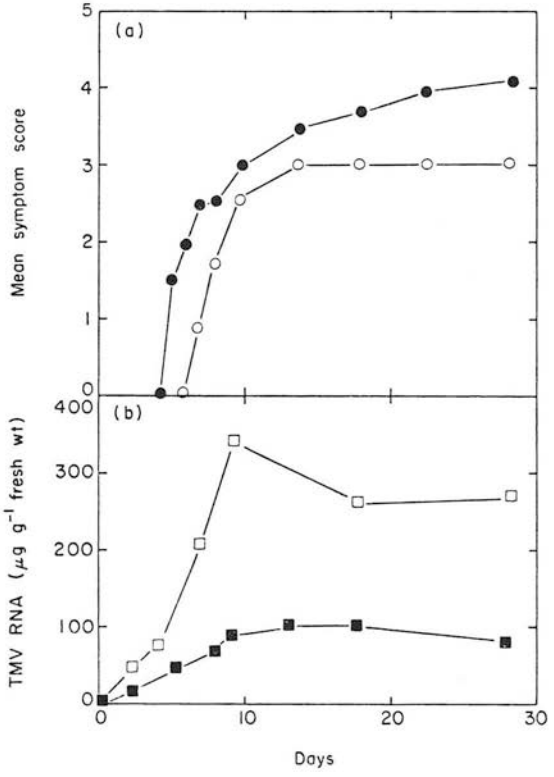

FIG. 2. Time courses of (a) symptom development and (b) TMV RNA accumulation in +/+ plants inoculated with TMV strain 0. Plants were grown at 29 °C (○, □) or 33 °C (●, ■) for 8 days before, and after inoculation.

In order to disentangle the effects of temperature on TMV multiplication from those of the *Tm-1* gene, the amounts of RNA accumulated in *Tm-1* hosts have been expressed as percentages of the amounts accumulated in comparable leaves of the +/+ host grown at the same temperature. Figure 1(b) shows that at 20 °C, resistance was highly effective, giving more than 95% inhibition of RNA accumulation in both *Tm-1* hosts. The inhibition of RNA accumulation in *Tm-1*/+ plants decreased with increasing growth temperature, until at 33 °C, the resistance gene had

little effect on accumulation. Converted to absolute values, the data of Fig. 1(a) show that the amount of RNA accumulated in *Tm-1*/+ plants at 33 °C (65 μg g^{-1}) was greater than at 24 °C (27 μg g^{-1}) or 20 °C (6 μg g^{-1}).

Tm-1/*Tm-1* plants also showed decreasing inhibition of TMV RNA accumulation with increasing temperature, but the inhibition was greater than in *Tm-1*/+ plants at all temperatures. This confirms our earlier suggestion that inhibition of TMV strain 0 multiplication is *Tm-1* gene dosage-dependent [*5*].

Effects of Tm-1 *on TMV strain 1 at different temperatures*

In susceptible plants, the severity of symptoms produced by strain 1 increased with temperature (Table 1). In this respect, strain 0 and strain 1 behaved similarly in +/+ plants. Strain 1 also caused symptoms of *Tm-1*/*Tm-1* plants, but these were not as severe as those caused on +/+ plants. In particular, only very mild symptoms were noted on *Tm-1*/*Tm-1* plants grown at a constant 20 °C.

TABLE 1

Accumulation of TMV RNA, and severity of mosaic symptoms caused by TMV strain 1, in tomato plants of different resistance genotypes grown at different temperatures

	Mean symptom score		TMV RNA concentration[a] (μg g^{-1} fresh wt)	
Host genotype:	+/+	*Tm-1*/*Tm-1*	+/+	*Tm-1*/*Tm-1*
Temperature (°C)				
20	2·0	0·2	*c*	*c*
25	3·0	2·2	267±13[b]	64±8
33	4·9	3·3	14±2	16±3

[a] Measured in systemically-infected leaves 19 days after inoculation. Similar results were obtained for directly-inoculated leaves.
[b] Means±standard errors of 6 replicate determinations.
[c] Not determined.

At 25 °C, strain 1 multiplied to high concentrations in +/+ plants, but its multiplication in *Tm-1*/*Tm-1* plants was inhibited by about 75%. This confirms our previous finding that this isolate of strain 1 does not completely overcome the inhibitory effect of the *Tm-1* gene on virus multiplication under normal conditions [*6*].

At 33 °C, strain 1 multiplication in +/+ plants was very low, attaining only 5% of the TMV RNA concentration reached at 25 °C. Thus, its multiplication was much more strongly inhibited by the effects of high temperature than was strain 0, which at 33 °C reached 57% of its concentration at 24 °C. By examining a series of TMV mutants, Jockusch [*9*] established a strong correlation between temperature-sensitivity of the coat protein subunit, and inability to multiply in plants grown at high temperature. Temperature-sensitive coat protein subunits are denatured *in vivo* and much of the TMV RNA which is synthesized is degraded because it is not encapsidated. Our strain 1 isolate has temperature-sensitive coat protein subunits, with a half life of denaturation at 32 °C of 47 min, compared with over 8 h for strain 0 coat protein (unpublished results). This is, therefore, a likely explanation for the low multiplication of strain 1 at 33 °C.

In *Tm-1*/*Tm-1* plants at 33 °C, strain 1 accumulated to almost the same concentration as in +/+ plants and an earlier sampling at 14 days, before necrosis had appeared on +/+ plants, also showed equal concentrations in the two genotypes. Thus, although the direct and strongly inhibitory effect of high temperature on strain 1 multiplication makes interpretation difficult, the data are not inconsistent with the hypothesis that strain 1 can overcome the inhibitory effects of the *Tm-1* gene on multiplication at high temperature.

Temperature shift experiments

To test how quickly the inhibition of TMV multiplication by *Tm-1* could be restored, plants were grown at 33 °C for 7 days before, and then for 8 days after inoculation with strain 0, to allow virus accumulation. They were then shifted to 23 °C where inhibition of TMV RNA accumulation would be expected in *Tm-1* plants. Figure 3 shows that both in susceptible plants, and in plants with the 2 resistance genotypes, there was a rapid fall in RNA concentration immediately after transfer to the lower temperature. This was not due to a rapid uptake of water on shifting to 23 °C since, in a typical experiment, dry weight only fell from 16 to 15% of fresh weight in the 2 days after transfer. Nor was there sufficient leaf growth in these 2 days to explain the reduction in RNA concentration. The conclusion therefore must be that in

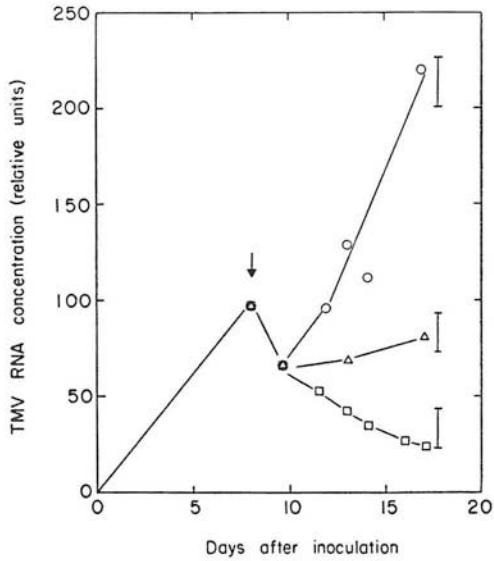

FIG. 3. Changes in TMV RNA concentration in tomato plants of different resistance genotypes. Plants were grown at 33 °C for 7 days before and 8 days after inoculation with TMV strain 0. At day 8, indicated by the arrow, plants were transferred to 23 °C for the remainder of the experiment. ○, Genotype +/+; △, genotype *Tm-1*/+; □, genotype *Tm-1*/*Tm-1*. To allow comparison of results from different hosts, values for each host are expressed relative to a value of 100 units at day 8. Absolute concentration of TMV RNA (μg g^{-1} fresh wt) at day 8 were: +/+, 71±6; *Tm-1*/+, 91±8; *Tm-1*/*Tm-1*, 11±2. Points are means of 4 to 8 replicate determinations. Vertical bars indicate the least significant difference (P=0·05) between points for each host

both susceptible and resistant hosts, some of the RNA previously made at 33 °C was degraded on transfer to the lower temperature. Although TMV is normally considered to be an extremely stable virus, it is possible that some of the virus made at the high temperature was defective and unstable.

In +/+ hosts, net TMV RNA accumulation was quickly restored and continued at a high rate for several days, while in *Tm-1*/*Tm-1* plants, there was a continued net loss of RNA for the remainder of the experiment. In *Tm-1*/+ plants, after the initial decrease in RNA concentration on shifting to the lower temperature, there was a slight increase for the remainder of the experiment.

A surprising feature of these temperature shift experiments was that *Tm-1* plants infected with strain 0 did develop mosaic symptoms when transferred to the lower temperature. Symptom expression was, however, transitory, as after further time at 23 °C, the plants increasingly produced symptom-free leaves, with consequent reduction in mean symptom score (Fig. 4).

When leaves of *Tm-1*/*Tm-1* plants, showing mosaic symptoms after transfer to 23 °C, were used to inoculate further *Tm-1* plants which were then kept at 23 °C, the latter plants developed no mosaic symptoms. Thus the mosaic symptoms developed by the plants shifted from 33 to 23 °C were not caused by strain 1 mutants arising during the high temperature growth period.

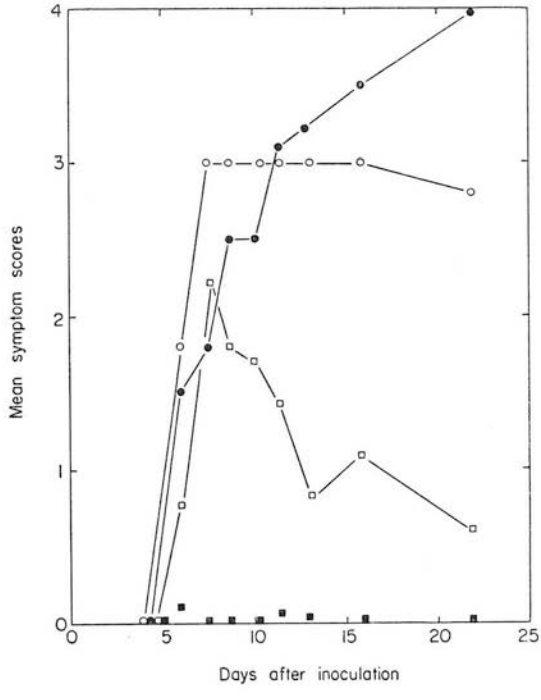

FIG. 4. Changes in mean symptom score in tomato plants of two genotypes grown under different temperature regimes. ○, ●, Genotype +/+; □, ■, genotype *Tm-1*/*Tm-1*. All plants were kept at 33 °C for 7 days before inoculation with TMV strain 0. (●, ■) Plants kept at 33 °C after inoculation, (○, □) plants kept at 33 °C for 5 days after inoculation, then transferred to 23 °C for the remainder of the experiment.

DISCUSSION

In this paper we have shown that the severity of symptoms formed by TMV strain 1 on *Tm-1* plants was highly temperature-dependent, with quite severe symptoms developing at high temperatures but very mild symptoms at low temperature. This is consistent with the results of Cirulli & Ciccarese [*2*], who tested the ability of a number of TMV isolates to cause symptoms on *Tm-1* plants, and found that the percentage of virulent isolates increased with temperature.

The *Tm-1* gene completely prevented the formation of mosaic symptoms by TMV strain 0 at all temperatures tested, and was thus temperature-independent in its action. In contrast, inhibition of multiplication of strain 0 and 1 by *Tm-1* was strongly temperature-dependent, decreasing with increasing temperature. Any hypothesis about the mechanism of action of *Tm-1* will have to explain this apparent independence of the effects of the gene on symptoms and multiplication.

When *Tm-1* plants infected with strain 0 were transferred from a high temperature, permitting virus multiplication, to a lower temperature where *Tm-1* inhibits multiplication, the results indicated a rapid restoration of the inhibitory activity. This may have represented rapid synthesis of new *Tm-1* product, or merely a resumption of activity by *Tm-1* product previously present but inactive at 33 °C.

The continued loss of TMV RNA concentration in *Tm-1*/*Tm-1* plants after transfer to 23 °C was not necessarily due to the activity of a *Tm-1* product causing degradation of existing virus, since net degradation of TMV RNA was also found in +/+ plants immediately after transfer. However, an activity of the *Tm-1* product in degradation of existing virus or TMV RNA cannot be excluded.

The transitory phase of mosaic symptoms caused on *Tm-1* plants after transfer to 23 °C may have been caused by the degradation products from virus destroyed after transfer. This result does show that in unusual circumstances, it is possible for TMV strain 0 to overcome the symptom suppression function of the *Tm-1* gene.

We thank Dr Tim Hall, Glasshouse Crops Research Institute, for useful discussions and for tomato seeds of isogenic lines and virus strains.

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Printed in Great Britain by Henry Ling Ltd., at the Dorset Press, Dorchester, Dorset

VIROLOGY 118, 263-266 (1982)

Does Tobacco Mosaic Virus RNA Contain Cytokinins?

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Received November 24, 1981; accepted January 5, 1982

Tobacco mosaic virus was previously reported to contain between 5 and 17 modified nucleosides with cytokinin activity per RNA molecule. Using a new and highly sensitive method for gas chromatography of permethylated cytokinins, we were unable to detect cytokinins in enzymic or alkaline digests of TMV RNA. The method was sensitive enough to assay 1 cytokinin nucleoside per 10 TMV RNA molecules. Soybean callus bioassay also failed to detect activity in TMV RNA hydrolysates which corresponded to any of the cytokinin nucleosides known to occur in RNA. We therefore suggest that TMV RNA does not contain cytokinin nucleosides.

The cytokinins, N^6-substituted derivatives of adenine, are plant hormones important in control of growth, morphogenesis, and senescence (*1*, *2*). They are also minor constituents of specific transfer RNAs from a wide range of organisms (*3*, *4*). Recently, they have been reported in tobacco mosaic virus (TMV) RNA (*5*, *6*). Bioassay of enzymically hydrolysed TMV RNA revealed eight fractions with cytokinin-like activity, including peaks tentatively identified as zeatin and isopentenyl adenine, and their respective derivatives. Quantitatively, the total cytokinin activity was approximately 5 to 17 residues per TMV RNA molecule (*6*). The biological significance of these cytokinin nucleosides, and their relationship to virus multiplication and pathogenesis are not known. We report here a reexamination of the cytokinin nucleosides in TMV RNA using a newly developed gas chromatographic assay which is highly sensitive and quantitative.

TMV strain U1 was multiplied in *Nicotiana tabacum* L. cv. Samsun and purified by polyethylene glycol precipitations (*7*). TMV RNA was extracted from purified virus by a detergent/phenol procedure and further purified by successive reprecipitations (*8*) before dialysis against water. Electrophoresis of the purified TMV RNA showed a single peak of molecular weight 2×10^6, with no detectable traces of other components.

For enzymic hydrolysis to nucleosides, 50 mg TMV RNA in 50 ml of 10 m*M* Tris-HCl, pH 7.8, was treated with 10 μg/ml pancreatic ribonuclease for 20 hr at 37°. The digest was adjusted to pH 8.4 and 10 m*M* $MgCl_2$, and treated with 2 μg/ml alkaline phosphatase for 8 hr. Snake venom phosphodiesterase was then added to 0.15 μg/ml. After a further 16 hr, the digest was heated to 60° for 30 min, then adjusted to pH 7.8. Nucleosides were extracted by partitioning five times with equal volumes of water-saturated *n*-butanol. Butanol extracts were reduced to dryness and redissolved in a small volume of 80% methanol for paper chromatography.

In a separate experiment, 50 mg TMV RNA was hydrolysed to nucleotides by treatment with 4 ml of 1 *M* NaOH for 4 days at 20°. After diluting to 50 ml and adjusting the pH to 8.4, the digest was treated with alkaline phosphatase in the presence of 10 m*M* $MgCl_2$ as above, to give nucleosides which were partitioned with butanol.

Enzymic or alkaline hydrolysates of TMV RNA were purified by chromatog-

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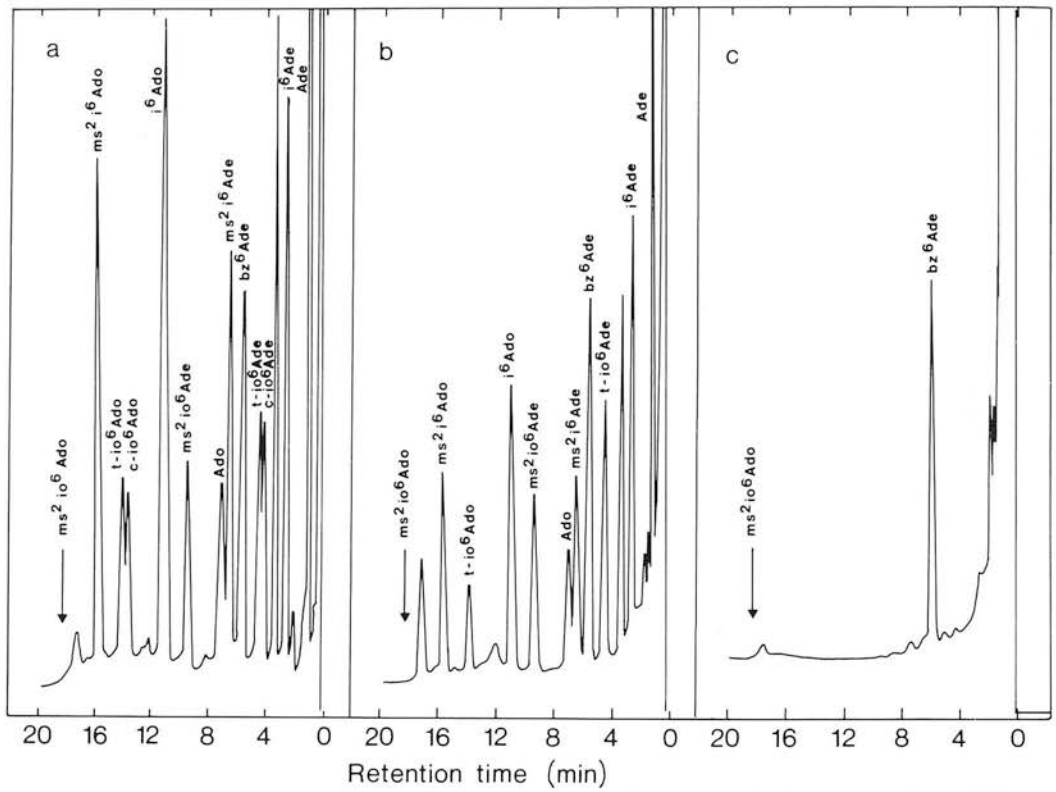

FIG. 1. Gas chromatograms of permethyl derivatives of cytokinin standards and hydrolysed TMV RNA. (a) 0.5 nmol of each cytokinin standard. (b) One-fiftieth of a sample containing 25 nmol of each of a series of cytokinins, which was alkali hydrolysed and chromatographed as explained in the text. (c) One-fiftieth of a sample of 50 mg TMV RNA after alkaline hydrolysis. The arrow indicates the calculated retention time of ms^2io^6Ado. Abbreviations used: i^6 = N^6-isopentenyl; io^6 = 6-(4-hydroxy-3-methylbut-2-enyl)-; c and t = *cis* and *trans* isomers; ms^2 = 2-methylthio-; Ade = adenine; Ado = adenosine; bz^6 = 6-benzyl. (Thus io^6Ade = zeatin; io^6Ado = zeatin riboside; and bz^6Ade = benzyladenine, the internal standard.)

raphy on prewashed 3MM paper developed with *n*-butanol/concentrated ammonium hydroxide/water (86/5/14, v/v). This chromatographic system completely separates the cytokinins known to occur in tRNA or reported in TMV RNA (R_fs between 0.6 and 0.9) from the four major RNA nucleosides (R_fs between 0 and 0.3). R_f zones 0.5 to 1.0 were eluted with 80% methanol and eluates further purified by thin-layer chromatography on silica gel plates developed with dichloromethane/methanol (85/15, v/v). Zones on chromatograms with mobilities corresponding to those of known cytokinin standards were eluted with methanol and dried *in vacuo* over phosphorus pentoxide.

Cytokinins were measured by gas chromatography using an alkali flame ionisation detector which is selective for nitrogen-containing compounds. Volatile derivatives of cytokinins suitable for gas chromatography were prepared by permethylation of extracts with dimethylsulphinyl carbanion in dimethyl sulphoxide (*9*, *10*). Benzyladenine was used as internal standard. The permethyl cytokinins were separated on a 2-m × 3-mm i.d. glass column of 1% OV-17/Gas Chrom Q, 100–120 mesh, temperature programmed from 220 to 300° at 5°/min. The detector was calibrated by chromatography of known amounts of individual permethyl cytokinin standards prepared under the same conditions. The different cytokinins are referred to by the abbreviated nomenclature of Skoog and Schmitz (*1*) as described in the Legend to Fig. 1.

All the cytokinins tested were clearly separated on the column: permethyl cytokinin bases eluted with retention times between 2 and 10 min, and the corresponding ribosides between 10 and 17 min (Fig. 1a). Adenine and adenosine were present as trace contaminants in the cytokinin standards. Standard ms^2io^6Ado was not available; an approximate retention time of 18 to 19 min for this compound was calculated by comparison with the other cytokinins. *cis* and *trans* isomers of io^6Ade and its derivatives had very similar retention times (Fig. 1a). The detection limit for individual cytokinins, based on a peak height twice background, was 1 ng.

Mixtures of cytokinin standards (25 nmol each of i^6Ado; ms^2i^6Ado; t-io^6Ado; and the corresponding free bases together with ms^2io^6Ade) were subjected to the same hydrolytic, partitioning, and chromatographic procedures as used for TMV RNA. Twenty-five nanomoles is equivalent to one cytokinin residue per TMV RNA molecule in a reaction mixture containing 50 mg TMV RNA. Gas chromatograms of the cytokinin standards were very similar before and after alkaline (Figs. 1a, b) or enzymic hydrolysis (not shown). Overall recoveries of i^6Ado, io^6Ado, and ms^2i^6Ado were 43, 48, and 40%, respectively; recoveries of the cytokinin bases were rather higher. These losses during treatment are normal for procedures of this type, and were probably due to physical factors such as incomplete elution from chromatograms and handling losses. We conclude that the cytokinin standards were substantially unaltered by the hydrolysis and purification procedures.

Gas chromatograms of alkaline hydrolysates of TMV RNA showed a large peak due to the internal standard permethyl benzyladenine (Fig. 1c). No peak was found in this region when the internal standard was omitted. No peaks with retention times corresponding to any of the cytokinin nucleosides were present above the detection limit of 1 ng. The small peaks on chromatograms, which had retention times different from cytokinin standards, were impurities eluting from the chromatography paper. A small peak with the same retention time as permethyl t-io^6Ade did not cochromatograph with authentic permethyl t-io^6Ade on further thin-layer purification. It, and also the later eluting peak at 17.5 min, were identified as contaminants present in the permethylation reaction mixture.

Figure 1c therefore suggests that cytokinin nucleosides do not occur in TMV RNA. A similar conclusion was reached when enzymically hydrolysed TMV RNA was analysed. It should be noted that the range of standard cytokinins fractionated by gas chromatography included all those previously tentatively identified in TMV RNA (*6*) as well as all those known in transfer RNA (*3*, *4*). The sensitivity of the method would have allowed detection of fewer than 1 cytokinin nucleoside per 10 TMV RNA molecules.

The failure to detect cytokinins in TMV RNA was most unlikely to have been due to incomplete hydrolysis of TMV RNA. As a control, wheat germ tRNA was hydrolysed under the same conditions. Gas chromatograms showed c-io^6Ado together with much smaller amounts of ms^2io^6Ado, i^6Ado, and ms^2i^6Ado, the cytokinins previously reported in this RNA (*11*, *12*). It is also unlikely that the cytokinin nucleosides were degraded during phenol extraction of TMV RNA. Wheat germ tRNA cytokinins are fully stable to phenol extraction (*11*), and phenol-extracted TMV RNA was used in the original investigations of its cytokinin content (*5*, *6*).

To confirm our results with gas chromatography, paper chromatograms of enzymic and alkaline hydrolysates of TMV RNA were also bioassayed using the soybean callus assay (*13*). No cytokinin activity was present on chromatograms at R_fs corresponding to those of the cytokinin standards (Fig. 2). The growth-stimulating activity at R_f 0.2–0.3 did not correspond to any known cytokinin nucleoside, and possibly resulted from the very high concentration of the major RNA nucleosides present in this region of the chromatogram.

Quantitative measurement of cytokinin activity by bioassay is difficult, as different cytokinins have different bioactivities (*14*). The bioassay is also less sensitive than gas chromatography; the detection

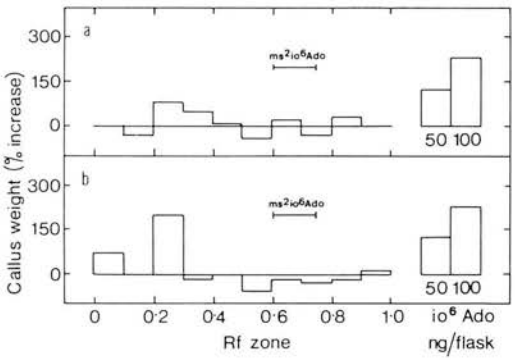

FIG. 2. Bioassay for cytokinin activity in (a) alkaline and (b) enzymic hydrolysates of 2.5-mg samples of TMV RNA. Callus cultures were grown on 30 ml nutrient agar in the dark at 25°. Callus growth, compared to blank paper controls, was measured 30 days later. R_f values of cytokinin nucleoside standards in this chromatographic system were i^{6}Ado: 0.80; c and t-io^{6}Ado: 0.64; ms^{2}i^{6}Ado: 0.80; the bar indicates an approximate R_f calculated for ms^{2}io^{6}Ado. The histograms indicate callus growth in reference flasks to which known amounts of io^{6}Ado standard were added.

limit for t-io^{6}Ado was approximately 20–50 ng. From the data in Fig. 2, it can be calculated that bioassay would have detected fewer than one cytokinin nucleoside per approximately three molecules of TMV RNA. Bioassay thus qualitatively confirmed the results obtained by gas chromatography.

Our results cannot exclude the possible presence of very low levels of cytokinin nucleosides in TMV RNA, less than 1 per 10 TMV RNA molecules. However, the biological significance of such a low level would be questionable.

ACKNOWLEDGMENT

We thank Dr. R. Horgan, University College of Wales, Aberystwyth, for the generous gift of cytokinin standards.

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PLANT GROWTH REGULATORS AND VIRUS INFECTION: A CRITICAL REVIEW

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Key words: Plant growth regulators, plant virus infection, control of host-virus interaction, resistance to virus disease, chemotherapy of virus disease

ABSTRACT

Virus infection can severely inhibit plant growth and distort development. This article reviews changes in plant growth regulator metabolism caused by infection. In general, virus infection decreases auxin and gibberellin concentrations and increases abscisic acid concentration. Ethylene production is stimulated in necrotic or chlorotic reactions to infection, but not where the virus spreads systemically without necrosis. While these broad trends are true for most host-virus combinations studied, several situations are recorded where the virus had other effects on growth substance concentration. Cytokinin changes do not show any common pattern: both increases and decreases after infection have been reported.

The extent to which virus-induced changes in growth substance concentration could be responsible for observed alterations in host growth and development is discussed. While changes in abscisic acid, gibberellin and ethylene production seem potentially important, the experimental evidence does not provide conclusive proof for control of growth by these changes.

The numerous investigations of effects of exogenous regulators on virus multiplication and pathogenesis are reviewed. Different regulators, or the same regulator applied at different times or concentrations had very diverse effects, and in some cases did significantly alter virus multiplication and pathogenesis. However, such studies seem to have yielded disappointingly little understanding of the biochemistry of host-virus interaction, and the possible involvement of growth substances in this.

Possible uses of plant growth regulators in chemotherapy of virus

disease, and their possible involvement in natural or induced resistance mechanisms are discussed.

INTRODUCTION

Viruses can cause serious inhibition of plant growth and loss of yield (21, 51, 90). Developmental processes may also be deranged, giving rise to distorted plants or bizarre growth forms such as tumours and phyllody (60, 75, 90). These alterations in host growth might be controlled in part by virus-induced alterations in growth regulator metabolism.

Some virus infections result in accumulation of very large amounts of viral nucleoprotein. For example, tobacco mosaic virus (TMV) can multiply in tobacco or tomato until it forms 1% of the leaf fresh weight (38, 44). In the synthesis of this vast amount of 'foreign' nucleoprotein, some 75% of the host capacity for synthesis of RNA and protein is diverted to production of viral products (39, 43). The means by which the virus takes over these anabolic activities of the host so effectively are not fully understood, but might involve alterations in controls of transcription and translation involving growth regulators.

Many cases are known where plants have genetically-controlled resistance to a virus normally infecting that species, or where apparent resistance may be induced in susceptible plants by a prior infection or chemical treatment. In no case do we have a complete understanding at the biochemical level of how these resistances operate, but early evidence indicates that plant growth regulators could be involved in certain resistance-like mechanisms.

An alternative approach to the study of virus infection and its interaction with growth regulator metabolism has been to alter the growth regulator metabolism of plants artificially by supply of exogenous regulators. Some of this work has been done in the hope of discovering chemical treatments which might interfere with the development of pathogenesis, and so be of potential use in the chemotherapy of virus disease.

In this article we review the effects of virus infection on metabolism of endogenous growth regulators, and discuss whether the changes could be the cause of altered host growth and metabolism. We consider the effects of exogenous regulators on virus multiplication and pathogenesis, and possible roles of growth regulating compounds in chemotherapy and resistance.

To our knowledge this is the only recent review of this subject. Other, earlier reviews (32, 80, 105, 121) have covered not only virus infections, but also the extensive literature on alterations in growth regulator metabolism caused by fungal and bacterial infections, which are beyond our scope here. Unlike viruses, fungi and bacteria may also produce or metabolize growth regulators themselves during infection.

EFFECTS OF VIRUS INFECTION ON ENDOGENOUS GROWTH REGULATOR CONCENTRATIONS

Plant viruses are very diverse in form. They range in size and complexity from the viroids; covalantly-closed circles of naked RNA with a sequence length of 350-450 nucleotide residues (54), to large complex particles such as clover wound tumour virus (WTV) containing up to 16 large, double-stranded RNAs and several types of protein (110). The majority are rod-shaped or isometric particles with a single type

of protein and a genome size of 5-15 kilobases (51).

The effects of virus infection on their host plants are equally diverse. Many, such as TMV and turnip yellow mosaic virus (TYMV) can spread systemically through the host and invade and multiply in virtually all living tissue. Others, such as tobacco necrosis virus (TNV) or tobacco rattle virus (TRV) are normally restricted to necrotic lesions which form around the infection sites. Some aphid-transmitted viruses are restricted to the phloem (139). With this great diversity of form and pathogenic effect, it is reasonable to expect that different viruses in different hosts could have very diverse effects on growth regulator metabolism. Furthermore, the extreme severity of symptoms caused by many viruses could give rise to changes in growth regulators which are secondary responses to the symptoms, and not directly involved in control of the host-virus interaction.

Measurement of endogenous growth regulator concentration in plants is difficult: most occur at very low concentration, in the presence of substances which interfere with the final assay. Many studies of the effects of virus infection of growth regulator metabolism have used bioassays. These methods tend to be only semi-quantitative, lack specificity and do not give unequivocal identification of the growth regulator being measured. If available, physical methods of assay such as gas chromatography or combined gas chromatography-mass spectrometry are much to be preferred, but these must be accompanied by adequate internal standardization and control experiments to establish that losses of growth regulators during extraction and purification are corrected for.

Auxins

The earliest experiments on effects of infection on growth regulators were with auxins in plants showing conspicuous stunting. Most reports indicated a reduction in auxin activity after infection, e.g. with tomato spotted wilt virus (TSWV) (53), potato leaf roll virus (PLRV) (61, 85, 130) and TMV (103, 104). Beet curly top virus (BCTV) reduced auxin concentration in susceptible lines of tomato, Phaseolus vulgaris and Beta vulgaris (127). In systemically infected tobacco, TMV decreased the concentration of indoleacetic acid (IAA) and phenylacetic acid (PAA) by up to 95%; concentrations of their precursors tryptophan and phenylalanine were increased (107). Dramatic reductions in leaf auxin content in these experiments were observed within 24 h of inoculation, when only a small proportion of leaf cells would be infected. If these results are confirmed, they would suggest that the reduction in auxin concentration is induced by some factor which spreads through the leaf faster than the virus.

One possible mechanism of reduced auxin activity after infection could involve increased activity of IAA oxidase, which has been reported in cowpea mosaic virus (CPMV) infection (79). Several other host-virus combinations have been shown to have increased activity of peroxidase, which has IAA oxidase activity (49, 83, 134, 149). There do not appear to have been any attempts to reverse virus-induced stunting in infected plants showing reduced auxin concentration by supply of exogenous auxin.

Infection has not only been reported to cause reduced auxin concentration. In barley yellow dwarf virus (BYDV)-infected barley, auxin concentration was unchanged although the infected plants were severely stunted (115). In lupins infected with pea mosaic virus (PMV) (151); potato in the initial stages of potato virus X (PVX) infection (62)

and tobacco forming local lesions after TMV infection (148), increased levels of auxin activity were found. Basipetal transport of ^{14}C-IAA was inhibited in stem sections from BCTV-infected tomato showing apical symptoms (128) although this was associated with detectable phloem damage (109). In contrast, an increased rate of auxin transport was found in stem sections of tomato after infection with aster yellows virus (AYV) (142) although this pathogen is now known to be a mycoplasma (51). It is very likely that effects of virus on long-distance movement of growth substances may be indirect, and non-specific in that rates of movement of other small molecules may be similarly altered.

Abscisic acid

The effects of virus infection on abscisic acid (ABA) concentration reported so far have been varied. Measurements of the β-inhibitor complex of growth-retarding substances, of which ABA is a major component (132), showed unchanged activity after TMV infection of tobacco (132), but increased activity in PMV-infected lupin (151) and rice tungro virus (RTV)-infected rice (95). In experiments where ABA was measured by physical methods, infection of cucumber with cucumber mosaic virus (CMV) was reported to cause no change (9), but other workers reported a three-fold increase (1).

A decrease in ABA concentration in TMV-infected tobacco was reported (107) but in this study only the very early stages of infection were examined. Other workers reported increases (10, 156). In our experiments (156), we used the White Burley variety of tobacco, containing the N' gene. This gives either a localized necrotic reaction, or allows systemic spread of virus, depending on the strain of TMV used. We found a two to six-fold increase in ABA concentration with systemic

infection, which persisted throughout the period of active virus multiplication. The local lesion strain of TMV caused a much larger increase in ABA, up to 20-fold. This increase was associated with the lesions rather than the interlesion areas, and first became detectable at the time of lesion appearance. The timing made it unlikely that ABA increase was a primary cause of lesion formation; it was more likely to have been a secondary consequence of necrosis.

Experiments in which ABA was sprayed onto healthy plants to produce an increase in internal concentration similar to that caused by systemic virus infection suggested that the virus-stimulated rise in ABA could be a major cause of the reduced growth of infected plants (156). Both TMV and ABA reduced leaf growth by inhibition of cell division, and had no effect on cell expansion. Experiments with the local lesion strain and radiolabelled ABA showed that ABA was transported from the necrotic, inoculated leaves to upper healthy leaves, in sufficient quantity to explain the observed inhibition of growth of the upper leaves (40). An analogous situation was reported by Fraser and Matthews (36), who found that inoculation of cotyledons of Chinese cabbage with TYMV caused a rapid transient inhibition of leaf initiation at the seedling apex, long before infectious virus could be shown to have invaded the apex. Although the translocated factor was not identified, ABA is a likely candidate.

The means by which TMV infection stimulates ABA production are not yet clear. In the necrotic reaction, it is possible that water loss from necrotic tissue and ABA release from disrupted chloroplasts is responsible. In systemic, non-necrotic infection, an early effect of TMV is to alter chloroplast metabolism, including chloroplast protein synthesis, chloroplast ribosomal RNA synthesis and the properties of chloroplast membrane proteins (37, 59, 74). It is

possible that this could allow release of ABA from chloroplasts, thus de-repressing its further synthesis within the chloroplast (58), although the role of the chloroplast in ABA synthesis has recently been questioned (57).

Systemic TMV infection causes the plant to synthesize large amounts of TMV RNA, and also causes an early transient stimulation of cytoplasmic ribosomal RNA synthesis (39). Treatment of healthy or TMV-infected leaf discs with sufficient exogenous ABA to cause an increase in internal ABA similar to that caused by virus infection also stimulated synthesis of both host and viral RNAs (155). It may therefore be speculated that the increase in ABA concentration caused by TMV could play a role in stimulating the observed increases in RNA synthesis after infection. This report of stimulation of RNA synthesis by ABA is in contrast to most reports showing an inhibition. Most other studies however have been with embryonic tissue and in the presence of other growth regulators (20, 152).

Although ABA is biochemically one of the simpler growth regulators, its metabolism includes two complications which must be taken into account when assessing data from the effects of virus infection. First, it is readily metabolized to bound forms including the glucosyl ester (91) and other glycosides (92, 120). The metabolic relationships and controls of interconversions between the free acid and bound forms are not fully understood, but the concentration of bound ABA may exceed that of the free acid in the leaf (153, 156), so any study which measures only free acid may present an incomplete picture. Secondly, ABA may either be sequestered in the chloroplast, or released to other parts of the cell. The presence of these reservoirs of spatially or chemically sequestered ABA might mask metabolically

significant changes in active ABA concentration caused by virus infection. It is perhaps relevant that in tobacco grown under water stress conditions, and so having a high endogenous ABA concentration, TMV infection caused no further increase in ABA (R. J. Whenham, unpublished data).

Gibberellins

The visible, and often dramatic stunting of shoot growth associated with virus infection also led to studies of gibberellic acid content. In cucumber seedlings infected with CMV, hypocotyl elongation was inhibited. Bailiss (8) reported that this was associated with a reduction in concentration of gibberellins A1 and A3 but found no qualitative difference in gibberellins between healthy and infected plants. In another study of the same system, gibberellin content was also found to be reduced after infection (1), but this was accompanied by increases in ABA concentration (1) and in ethylene production (89). Ben-Tal and Marco (18) reported that there were qualitative changes in gibberellin content after infection, and suggested that the pattern of gibberelllin degradation was altered. They were unable to conclude whether the decreased gibberellin concentration was the cause of the stunting or merely a consequence of disease symptoms without a controlling role. Infection of barley with BYDV caused stunting and decreased gibberellin concentration (115).

Some reports have shown that virus-stunting may be partly prevented by treating plants with exogenous gibberellins, for example with severe etch virus (SEV) in tobacco (133), or with corn stunt virus (CSV), AYV and WTV infection (86). These experiments provide at best circumstantial evidence that virus-induced reduction in endogenous gibberellin concentration was responsible for stunting. Healthy cucumber seedlings were shown to respond more to applied gibberellin than CMV-infected, stunted seedlings (34, 87), raising the possibility

that although applied gibberellin may stimulate elongation, this may be by quite separate means from that by which virus infection inhibits elongation.

There have been several reports that inhibition of stem growth by virus infection is associated with a reduction in cell division rather than cell expansion (7, 27, 120, 158); application of exogenous gibberellin has been reported to stimulate cell division rather than cell expansion in some species, but both processes in others (reviewed in Reference 52).

Gibberellin concentration was reported to be unaltered by TMV infection of tobacco (22) and by tomato aspermy virus (TAV) in tomato (7).

Cytokinins

Tobacco ringspot virus (TRSV) infection of Nicotiana glutinosa or cowpea was reported to reduce cytokinin activity (71, 140). In contrast, TMV and CMV infections increased cytokinin activity in tobacco (137, 138). Chromatograms of extracts from both healthy and systemically infected plants showed peaks of cytokinin activity in bioassay, corresponding to zeatin and zeatin riboside. Activity of both was higher in the infected tissue, which also contained two cytokinin activities not present in healthy leaves. Inoculation of the tobacco cultivar Xanthi-nc, which restricts TMV to necrotic local lesions, was also reported to cause an increase in cytokinin activity (135).

All these studies used bioassay to quantify cytokinin activity. This has two shortcomings: accurate estimation of quantitative changes in cytokinin concentration after infection is difficult, especially as different cytokinins have different bioactivities in the assay system. Secondly, identification of the chemical nature of the cytokinin activity by bioassay of chromatographically-fractionated

extracts is far from unequivocal. There is a clear need for accurate estimation and characterization of cytokinin activity in healthy and infected tissue using rigorous physical methods.

An interesting and provocative connection between TMV and cytokinin was the suggestion the TMV RNA may itself contain residues with cytokinin bioactivity (135, 136). The major active components were tentatively identified by bioassay after paper or Sephadex LH20 chromatography as zeatin and isopentenyladenine and their respective derivatives. It was suggested that each TMV RNA molecule, 6340 nucleotide residues long, contained between 5 and 17 cytokinin-active nucleosides. As TMV RNA can reach 400 µg / g fresh weight in a successful infection (38), this represents a cytokinin concentration equivalent to 300 - 1050 ng / g; much higher that the normal leaf level of 1 - 10 ng / g (76) or the amount found in tRNA (55).

We have recently attempted to confirm the presence of cytokinin nucleosides in TMV RNA by a newly developed and highly sensitive gas chromatographic procedure, using permethylated cytokinins and a nitrogen-specific detector (154). We failed to detect any cytokinin nucleoside known to occur in RNA by this method or by soybean callus bioassay (157). As the gas-chromatographic method would have detected as little as 1 cytokinin nucleoside per 10 TMV RNA molecules, it suggests that cytokinins are either absent, or present in such small amounts as to be of questionable biological significance.

Ethylene

Generally, ethylene production appears to be increased by infection leading to necrotic or chlorotic reactions, but not by viruses which multiply systemically without necrosis. Ethylene production was stimulated 3 to 13 h before appearance of local lesions after TMV (106) and TNV (48) infection of tobacco, but the largest increases

occurred after lesions appeared. A sudden burst of ethylene has also been reported before TMV lesions (33). Physalis floridana leaves infected with potato virus Y (PVY) showed increased rates of ethylene production at the onset of necrosis (113), while cowpea leaves infected with CMV or CPMV also showed increased ethylene production (66, 79). Tobacco leaves systemically-infected with TMV did not have increased rates of ethylene production (15, 48, 98) except in very late stages of infection in senescent leaves (98). In beet, ethylene production was unchanged during leaf yellowing caused by infection with beet mosaic virus (BMV) and beet mild yellows virus (BMYV); in contrast a necrotic infection with the fungus Cercospora beticola caused increased ethylene production (70).

These observations raise the question of whether ethylene is actively involved in promoting the necrotic response; the evidence is contradictory. In tobacco reacting hypersensitively to TMV infection, an inhibitor of ethylene synthesis, succinic acid 2,2'-dimethyl-hydrazide did not alter lesion size (106). Ethylene production in TMV-infected leaves was also inhibited by treatment with aminoethoxy-vinylglycine, but lesions still formed (33). In Tetragonia expansa, bean yellow mosaic virus (BYMV) normally causes chlorotic lesions. These lesions became necrotic when plants were exposed to ethylene, while in those exposed to CO_2, an inhibitor of ethylene action, development of both chlorosis and necrosis was prevented (10). When tobacco leaves were pricked with needles moistened with the ethylene-releasing compound 2-chloroethylphosphonic acid (Ethrel, ethephon), necrotic spots similar to virus-induced lesions were produced (146). Changes in protein constitution and peroxidase isoenzyme patterns induced by ethephon and TMV necrosis were similar. Treatment of inoculated leaves with ethephon reduced the size of lesions formed (146). These

experiments provide evidence for a role of ethylene in control of the necrotic response, but do not prove that ethylene and TMV induce necrosis by the same mechanism. It seems likely that much of the ethylene production associated with virus-induced necrosis may be a consequence of non-specific wounding effects. Ethylene synthesis occurs at the plasmalemma (78); it is interesting that some of the early effects of virus infection include altered membrane permeability (74) and increased activity of a membrane-bound ATPase (64).

Virus-stimulated ethylene production appears to occur without necrosis in CMV-infected cucumber seedlings. Ethylene production increased at the time of appearance of chlorotic lesions on cotyledons (88). It was suggested that the increased ethylene production, coupled with an increase in resistance of cotyledons to gaseous diffusion, may have been responsible for the marked epinasty of cotyledons seen after infection. Exogenous ethylene also caused epinasty of cotylendons on healthy plants (77). Ethylene production was also shown to be enhanced in hypocotyls of infected seedlings; experiments with application of ethylene or ethephon to healthy seedlings suggested that the CMV-induced ethylene production could be important in the suppression of hypocotyl elongation after infection (89), but is probably not the sole cause. Removal of ethylene by oxidation with potassium permanganate only temporarily stimulated growth of infected plants (89).

Although most studies of changes in endogenous growth substances after infection have been concerned with individual growth substances, there have been some attempts to interrelate changes in several growth substances (87). Perhaps the best studied system is CMV-infection of cucumber. Inhibition of hypocotyl growth in infected plants was associated with decreased gibberellin content (1, 8); increased ABA content (1) and increased ethylene production (89), all of which could

have contributed to the stunting. A careful and intensive study of the timings of the changes, coupled with application of physiologically reasonable concentrations of exogenous growth regulators, might establish which changes were important in control of growth, and which were secondary effects. However, until we have a deeper understanding of how growth substances may interact in growth control in the healthy plant, the question will remain difficult to answer.

EFFECTS OF EXOGENOUS GROWTH REGULATORS ON VIRUS MULTIPLICATION AND PATHOGENESIS

We considered above some experiments where exogenous growth regulators were applied to healthy plants, to mimic changes in regulator concentration caused by virus, or applied to infected plants to rectify a virus-induced reduction in growth regulator concentration. These experiments were designed to test the involvement of virus-induced changes in regulator concentration in control of host growth. In this section we are concerned with the effects of exogenous growth regulators on virus multiplication and development of pathogenesis.

Senescence: ABA and ethylene

There have been several reports that susceptibility of plants to virus is increased by treatments which promote leaf senescence. Infiltration of tobacco leaf discs, or injection of attached leaves with ABA caused small increases in size and number of lesions resulting from infection with TMV, and a small increase in the amount of infectious virus produced (10, 14). Unfortunately, the data were not analyzed for statistical significance. The highest ABA concentrations used (10 and

100 µg / ml) were phytotoxic, and with these methods of application which favour high ABA uptake, probably caused increases in internal ABA concentration far beyond normal physiological concentrations (155). In a separate study, we found that spraying intact plants with ABA at a concentration sufficient to produce a physiologically reasonable increase in internal ABA concentration caused statistically significant reductions in lesion size and number after TMV infection (42).

Ageing tobacco leaves on intact plants do not accumulate high concentrations of ABA (156). Old, flowering plants of the cv. Xanthi-nc formed very many fewer lesions when inoculated than young plants (41). Multiplication of TMV in the systemic cv. Samsun was much greater in leaves inoculated when young than in those inoculated when mature and fully expanded (38). These results suggest that there is no correlation in the intact plant between senescence and susceptibility to infection, and that the apparent increased susceptibility by treatment with high ABA concentration (10, 14) may have been non-specific effects of chemical damage, bearing little relation to any action of ABA *in vivo*.

Treatment of Xanthi-nc tobacco leaves with ethrel caused changes similar to senescence, and was reported to increase susecptibility to TMV infection, measured variously as small increases in lesion number, diameter and TMV multiplication (10, 13). In a separate study, treatment with ethylene did not increase TMV multiplication or lesion formation (98). A third report showed that ethephon treatment reduced lesion size (145, 146). Ethrel did not increase TMV multiplication in the systemic host *Physalis floridana* (25). The weight of the evidence is therefore against any increase in susceptibility to virus infection after ethylene-induced senescence.

Changes similar to senescence may also be induced by detaching leaves. The effects on susceptibility to TMV were complex, but did not provide consistent evidence that induced senescence enhances susceptibility to TMV (97, 99).

Exogenous regulators and the local lesion response

Many investigators have sought to analyze the interaction of exogenous growth regulators with virus multiplication and host response by studying the effects of regulators on local lesion formation. This experimental system yields data readily, but is biologically extremely complex; consequently the effects reported are varied and somewhat contradictory.

In Samsun NN tobacco, a local lesion host for TMV, treatment with high (near phytotoxic) concentrations of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) greatly promoted lesion expansion, while lower doses inhibited expansion (123, 147). Infected protoplasts from tobacco plants with the N-gene for local lesion formation did not become necrotic (101). Cultured in the absence of 2,4-D, protoplasts from N-gene plants supported much lower virus multiplication than those from systemic hosts lacking the N-gene (82). However, addition of 2,4-D at 1 µg / ml, the normal concentration used in protoplast culture, increased the amount of virus infectivity produced in protoplasts from N-gene plants, and reduced it in those from non-N-gene plants, so that they were equal. The difference between the two genotypes, and the differential effect of 2,4-D, were apparently eliminated in protoplasts from plants which had received high levels of nutrient, or those which were inoculated with a high virus concentration. These factors, and the effects of a range of 2,4-D concentrations, would require further study before it can be concluded that 2,4-D is interacting with the mechanism normally inhibiting

virus spread in N-gene plants. Treatment of cucumber with 2,4-D increased the number of lesions formed on inoculation with TMV (18).

TMV lesion size on young expanding leaves of N-gene tobacco plants was also reduced by other synthetic (α-naphthylacetic acid; NAA) and natural auxins (IAA, PAA) (147). Unlike 2,4-D, these compounds did not cause increased lesion size at high concentration. Auxins also failed to restrict lesion size in older leaves, detached leaves, or those with the 'acquired systemic resistance' phenomenon (112). Clearly the interaction of auxin with other factors in leaf development and metabolism is complex and influences its ability to alter lesion development. It has been suggested that auxin-stimulated ethylene production could be responsible for inhibition of lesion growth (147).

Reports of exogenous cytokinins altering lesion development are legion. Kinetin reduced lesion number and virus infectivity produced in TMV-infected discs of N. glutinosa (67). With the same host, others reported that lesion formation was inhibited but that virus production was stimulated by various cytokinins (93). The latter workers also reported that lesion growth was either inhibited or stimulated by two particular cytokinins. Aldwinckle subsequently pointed out that these were both benzyladenine masquerading under two synonyms(3)! Kinetin at various concentrations was reported to increase both size and number of lesions formed on detached leaves (35); preliminary experiments indicated that zeatin had a similar effect.

In TMV-infected Xanthi-nc tobacco, kinetin did not reduce the number of local lesions, but did reduce their necrotisation to the extent where they became invisible to the naked eye (17). Virus multiplication in contrast was not inhibited by kinetin. Kasamo and Shimomura (63) suggested that the epidermis and underlying tissue of tobacco leaves have different responses to kinetin as it affects the necrotic reaction.

As TMV initially multiplies in epidermal cells then spreads later to the mesophyll, they suggested that this might explain the different effects reported, especially the effects of time of application which are discussed in more detail below. In N. glutinosa and Datura stramonium, application of cytokinins both increased and decreased lesion number, depending on conditions (116).

Kinetin reduced the size and number of lesions caused by TSWV on petunia leaf strips and greatly reduced multiplication of this virus in tomato leaves (118). Benzyladenine reduced lesions on petunia and N. rustica caused by TSWV. Supplied before inoculation of N. rustica it reduced production of virus infectivity; if supplied after inoculation it increased it (4).

In cowpea, kinetin reduced the number of lesions formed by TRSV by 75% (71). Infectivity of the same virus produced in N. glutinosa was markedly reduced if plants were treated with high concentrations of kinetin 9 days before inoculation but not if treated 3 days before (140).

The general conclusion from these results is that while the most common effect of cytokinin appears to be to reduce lesion number and size, this may or may not be associated with a reduction in virus multiplication within the lesion, i.e. there may be an inhibition of necrotisation rather than of virus multiplication. Time of application relative to inoculation appears to be important in determining the effect of cytokinins and could be the cause of their ability to increase as well as decrease lesion number and size. There seem to be no reports as yet of cytokinins allowing a virus normally restricted to local lesions to spread systemically.

Exogenous regulators and the systemically-infected host

A less complicated approach to study the effects of exogenous growth regulators on virus multiplication and pathogenesis has been to

apply them to plants which permit systemic multiplication of virus. The results are then unobscured by the complex inhibitory effects of the local lesion reaction on virus multiplication.

The auxins NAA and indolebutyric acid (IBA) retarded appearance of TMV mosaic symptoms on tobacco, but only when applied at near-phytotoxic concentrations (100). These auxins were also reported to inhibit TMV multiplication in tissue culture (72, 73). IAA reduced TMV multiplication in young, expanding tobacco leaves, but only by about 30% (147). In contrast, 2,4-D was reported to stimulate TMV multiplication in Physalis floridana (25) and in tobacco (117). The latter author also reported that IAA increased TMV multiplication. TMV normally causes only a subliminal infection in cotton: an interesting report was that 2,4-D and several other growth regulators considerably increased the level of multiplication (26).

Kinetin either increased or decreased the infectivity of tomato aucuba mosaic virus (TAMV) produced in tobacco leaves, depending on the time of application (30). The most effective suppression was when kinetin was supplied from 4 weeks before inoculation. On a much shorter time scale, application of benzyladenine to tobacco leaves before or 1 min after inoculation with TMV reduced the production of infectivity; application 5 - 120 min after inoculation enhanced infectivity (3). Kinetin, kinetin riboside, isopentenyl adenosine and benzyladenine all increased the infectivity of TMV produced in systemically-infected tobacco or N. rustica plants (93), but all reduced production of infectivity in tissue cultured from infected plants (94). In CMV-infected meristematic cultures of N. rustica, kinetin at various concentrations had little effect on infectivity, or slightly stimulated it (124). Low concentrations of kinetin or benzyl-adenine increased the TMV infectivity produced in tomato leaf discs; high concentrations reduced it (11). Kinetin increased TYMV multiplication

in floated leaf discs of Chinese cabbage (19) but inhibited TMV and PVX multiplication in tobacco and Datura stramonium (111).

The effects of exogenous auxins and cytokinins on multiplication of viruses causing systemic infection are therefore very varied; clearly concentration and time of application are again major factors determining the effect. It should be noted that in no case has there been any demonstration of an effect of the growth regulator directly on the virus replication process as such, though generally these effects have not been sought in suitable in vitro experimental systems. Ralph et al. (108) found no effect of zeatin or benzyladenine on initiation of protein synthesis or rate of polypeptide chain elongation in a wheat germ cell-free system programmed with TMV RNA. It may be that the effects of exogenous auxins and cytokinins on virus multiplication are indirect consequences of primary effects on other aspects of host metabolism.

Some general comments on the design of experiments to study the effects of auxins and cytokinins on local lesion and systemic virus infections are pertinent. The majority of tests have measured virus multiplication by infectivity bioassay, by measuring the number of local lesions produced on a suitably reacting second host. This has some disadvantages: without considerable elaboration, the method is not really accurate enough to discriminate small differences in virus concentration, yet many of the reported effects of exogenous growth regulators are indeed small. With a few exceptions, much of the published work does not include proper statistical analysis, with appropriate transformation of raw data (68). Furthermore, treatment of plant tissue with growth regulators might well have an indirect effect on the specific infectivity of the virus produced (number of lesions induced per unit weight or virus),

especially at high or phytotoxic concentrations. Thus a reduced infectivity in crude extracts might not reflect a reduction in virus concentration. For these reasons some physical measurement of virus multiplication is desirable before claiming an effect of a growth regulator on multiplication.

Many experiments with exogenous auxins and cytokinins have been done with potentially unphysiological concentrations, but generally there have been no measurements of amounts of uptake or alteration in internal concentration caused by treatment. The vast majority of experiments with cytokinins have used kinetin and benzyladenine, which do not occur naturally in leaves, and whose metabolism might perhaps be quite different from naturally-occurring compounds. It would perhaps be interesting to test the forms of cytokinins occurring naturally in the tissue being studied.

Many experiments have been done with detached leaves or leaf discs. While this is a useful experimental approach, excision in itself does induce metabolic changes, especially in long-term experiments (2). These might influence the response of tissue and virus to exogenous growth regulators and make conclusions inapplicable to the intact plant.

Chemotherapy

The ability of exogenous growth regulators to delay or reduce the severity of visible symptoms of virus infection, and in some cases to inhibit virus multiplication, has stimulated research on possible methods of chemotherapy. One approach was to screen chemicals already in use in agriculture, which were known to be taken up and to spread systemically. The fungicide thiabendazole (TBZ) reduced the susceptibility of sugar beet to yellowing viruses (114). Part of the effect was attributed to a reduced

ability of the aphid vectors to colonise treated plants, but an effect on susceptibility of treated plants to virus was also possible. Methyl benzimidazol-2yl carbamate (MBC), the water decomposition product and fungitoxic principle of the benomyl fungicides (28), inhibited formation of visible symptoms of TMV on tobacco and beet western yellows virus (BWYV) in lettuce (143). Other studies showed that the multiplication of TMV in tobacco was strongly inhibited (46), and that MBC treatment completely prevented the normal inhibition of plant growth resulting from TMV infection (47). MBC doses giving over 90% inhibition of TMV multiplication were about one-fiftieth of those required to produce phytotoxic effects and growth inhibition (47). However, the multiplication of virus was inhibited, not abolished, and MBC-treated plants did eventually accumulate high concentrations of TMV after several months.

Both TBZ and MBC have activity in cytokinin bioassays (125, 141). We found that MBC had no direct inhibitory effect on TMV RNA synthesis. It was ineffective in suppression of TMV multiplication when supplied to mature leaves, but caused a persistent inhibition of TMV multiplication lasting well through leaf maturity and senescence, if supplied to the leaf while still very young. This suggests that the inhibitory effect of MBC on TMV multiplication was an indirect one through altered leaf development (47). One possibility is that the cytokinin activity of MBC maintains the leaf in some juvenile state unsuitable for virus multiplication. In many cases juvenile tissues such as meristems are known to support little or no virus multiplication (29, 129, 131). MBC was found to be ineffective against several other viruses (11, 143).

An alternative approach to chemotherapy has been to screen chemicals such as virazole (synonym ribavirin; 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide)(126) which have activity against animal viruses.

Virazole was effective in elimination of various viruses from plant tissue cultures (24, 122, 124) and reduced virus multiplication in intact plants (56, 69, 117). Its mode of action in plants is not understood, but it is known to have cytokinin-like activity; it stimulated growth of tissue cultures at low concentration and suppressed root formation (124). High concentrations of kinetin in the growth medium antagonised the antiviral action of virazole (124). Pulse labelling experiments failed to show any effect of virazole directly on TMV RNA or coat protein synthesis in infected tobacco protoplasts (R S S Fraser and A Gerwitz, unpublished data). These results raise the possibility that virus eradication by virazole might depend on a mechanism involving its cytokinin activity. The interaction of its antiviral activity with exogenous kinetin further suggests that knowledge of the effects of cytokinins on virus multiplication and virazole would be required before this type of chemotherapy could be fully exploited.

PLANT GROWTH REGULATORS AND RESISTANCE TO VIRUS DISEASE

Developmental or spatial resistance

In systemic infection, certain areas of the plant may be partially or completely resistant to invasion by the virus. Typically these areas include shoot and root meristems (29, 129, 131). Embryos or the entire seed may also not be invaded (102). No explanation is yet available for the apparent resistance of these tissues to infection; a possibility which deserves study is that it is related to their particular and unusual growth regulator status.

A related type of resistance is found when leaves are invaded at a

very early stage of development, for example with TMV or TYMV. Certain cells become infected, and give rise to yellow, diseased tissue as the leaf expands. Other groups of cells remain uninfected, and by division give rise to 'green islands' which remain substantially virus free (6, 81, 96). These green islands have higher chlorophyll concentration than healthy leaves, and may be analogous to meristematic tissue in that they are resistant to infection because they are suspended in some juvenile, non-infectible state. It has been reported that they have higher cytokinin content than the surrounding infected tissue (135). The possible role of endogenous growth regulators in maintaining the virus-free state of the dark green islands clearly deserves further investigation.

Constitutive resistance

Many cases are known where a particular variety of a species can contain a gene or genes conferring resistance or tolerance to a virus normally affecting that species. In no case do we have a full understanding of the biochemistry of gene action; superficially the various mechanisms appear to be very diverse.

BCTV caused auxin concentration to fall equally in susceptible and virus-resistant varieties of tomato, Phaseolus bean and beet, although the resistant varieties showed less severe symptoms and lower virus multiplication (127). In tomato, a TMV-tolerant variety was found to have higher concentrations of gibberellins and cytokinins in its sap than a susceptible variety (119). The relationship of this finding to the expressed tolerance is not clear, but in view of the experimental evidence that exogenous growth regulators can suppress symptoms and virus multiplication, a mechanism of tolerance based on endogenous growth regulators deserves further study.

Induced resistance

When plants form necrotic lesions after inoculation with virus, the response of uninoculated parts to a second or challenge inoculation can be altered. Lesions formed after the challenge inoculation are often smaller and less numerous than those formed on previously uninoculated control plants. This effect, referred to as 'induced' or 'acquired systemic resistance' has been reported in several host-virus combinations (112). In tobacco varieties forming lesions after TMV infection, at least four new host-coded proteins are detectable in parts of the plant showing acquired systemic resistance (50, 150). It has been suggested that these 'pathogenesis-related' proteins are involved in the resistance, perhaps in some way analogous to interferon in animals (65). However, whether these proteins play any part in the apparent resistance has recently been questioned (41, 42).

An alternative explanation of acquired resistance is based on changes in host growth regulator metabolism induced by the first inoculation, and proposes separate mechanisms controlling altered lesion number and lesion size in the second inoculation. Leaves showing acquired resistance were reported to have higher cytokinin content than control leaves (16, 137). The increases were not great, and the types of cytokinin involved were not identified, but the reports are consistent with other evidence that exogenous cytokinin can reduce lesion size by inhibiting necrotisation (12, 93, 118). This is supported by evidence that the amount of virus multiplication in small lesions on leaves with acquired resistance is as great as in the larger lesions of control leaves (16, 40).

Alteration in lesion number as a consequence of the first inoculation appears to bear an inverse relationship to induced changes in leaf abscisic

acid concentration. 'Resistant' leaves of N. tabacum formed fewer lesions in the second inoculation, and had higher ABA concentration at the time of inoculation than comparable leaves on control plants (40, 156). In experiments with radiolabelled ABA, it was shown that the increased ABA concentration of upper, uninoculated leaves was at least partly due to transport of ABA from the lower, lesion-bearing leaves. In N. glutinosa the converse situation applied. A primary inoculation of lower leaves with TMV reduced ABA concentration of uninoculated upper leaves, but increased the number of lesions they formed on subsequent challenge inoculation (45). The reduction in number of lesions formed when healthy N. tabacum plants were sprayed with ABA before inoculation is also consistent with this correlation (42). Whether ABA is the direct control of altered susceptibility to infection, or indirectly involved through an effect on leaf water relations and mechanical susceptibility to infection remains to be established. Van Loon (146) has shown that ethephon treatment induces effects similar to acquired systemic resistance and has suggested that ethylene production by lower, lesion-forming leaves could be responsible for the 'resistance' or upper leaves.

It is interesting in connection with the proposed growth regulator explanation of acquired systemic resistance that growth regulators have also been reported to induce synthesis of the pathogenesis-related proteins in healthy plants (5, 146).

CONCLUSIONS

There is considerable evidence that virus infection can cause major changes in growth regulator concentrations. Some of these changes have been

shown to be of potential importance in the control of host growth after infection; others appear to be secondary effects. There is very little information on how virus infection causes alteration in host growth regulator metabolism. The possible involvement of chloroplasts in synthesis or compartmentalization of some growth substances (31, 58, 84) and the observed early effects of infection on chloroplast membranes and metabolism (37, 59, 74) suggest further lines of study.

There is little understanding of how virus-induced changes in host growth regulators might cause alterations in growth, development and metabolism. This point reflects the paucity of our current knowledge of how growth regulators work in healthy plants (144). Virus infections may indeed by a useful experimental approach to the study of growth regulator function and metabolism.

Experiments with exogenous growth regulators and infection have suggested that they can influence virus multiplication and pathogenesis. To date they have given disappointingly little insight into how growth regulators may affect multiplication and symptom development. Early work on chemotherapy and involvement of growth regulators in mechanisms of resistance raises the possibility of ultimately developing new antiviral strategies based on manipulation of endogenous growth regulators.

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Eur. J. Biochem. *34*, 153—158 (1973)

Binding of Radioactive Homopolynucleotides to RNA

A Simple Method for the Detection, during Gel Electrophoresis or Sedimentation, of Messenger RNAs which Contain Poly(A) Sequences

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(Received November 9, 1972)

A simple method is described for the characterisation and assay of messenger RNAs which contain polyadenylic acid sequences. Total RNA is mixed with radioactive polyuridylic acid or polythymidylic acid, which bind to polyadenylic acid sequences. When the mixture is fractionated by gel electrophoresis or sedimentation, the radioactivity profile then shows the distribution of those RNAs which contain polyadenylic acid sequences. The amount of homopolynucleotide bound is a measure of the amount of messenger RNA present. The detection and assay of 10-S haemoglobin mRNA in mouse-reticulocyte total RNA is shown as an example of the technique.

Sequences rich in adenylic acid residues have been found in several mammalian messenger RNA species, such as in the rabbit haemoglobin mRNA [1], in HeLa cell mRNA [2] and nuclear heterogeneous RNA [3]. Several methods have been developed for the physical separation of mRNAs with contain poly(A) sequences from ribosomal and other RNAs [4—6].

We describe a method for determining the occurrence and size of RNAs which contain poly(A) sequences. The simplest possible approach to such an analysis is to mix radioactively labelled polyuridylic acid or polythymidylic acid with unlabelled total RNA under conditions favouring binding of the homopolynucleotides to poly(A) sequences. The mixture is then fractionated by sucrose gradient sedimentation or gel electrophoresis and the distribution of radioactivity in the fractions is determined. This paper describes experiments which use the mRNA for haemoglobin in mouse-reticulocyte total RNA as a model for the detection of messenger.

MATERIALS AND METHODS

Preparation of Mouse-Reticulocyte RNA

Frozen mouse reticulocytes (kindly given by Dr R. Williamson) were thawed into at least ten volumes of a detergent-lysing medium containing 2% (w/v) of sodium triisopropylnaphthalenesulphonate (Eastman Organic Chemicals), 0.1 M NaCl and 0.01 M Tris-HCl pH 7.5 at room temperature. The mixture was shaken with an equal volume of the phenol-cresol mixture of Parish and Kirby [7] and extraction of nucleic acid then followed the procedure described by Loening *et al.* [8]. DNA was not removed.

Homopolynucleotides

Poly([^{3}H]uridylic acid) (0.2 μCi/μg) was purchased from Miles Laboratories Inc. and was used dissolved in 0.5% sodium dodecylsulphate, 150 mM sodium acetate pH 6.0 at a concentration of 0.1 or 0.01 μg/μl. The minimum molecular weight of the poly(U) was 50000.

Polythymidylic acid was purchased from Miles Laboratories Inc. and was terminally labelled with [^{32}P]phosphate by the method of Richardson [9]. 5 A_{260} units of poly(dT) were dissolved in 50 mM Tris-HCl, 10 mM $MgCl_2$ and digested with 100 μg alkaline phosphatase (Whatman, from *Escherichia coli*) for 30 min at 37 °C. The enzyme was removed by one extraction with phenol and the poly(dT) precipitated with alcohol. The precipitate was dissolved in 10 mM Tris-HCl pH 7.4, 20 mM NaCl, 5 mM $MgCl_2$ and labelled by addition of [γ-^{32}P]ATP (Radiochemical Centre, Amersham, U.K.) and excess polynucleotide kinase (from *Micrococcus lysodeikticus*, kindly given by Dr K. Murray) for 45 min at 37 °C. An equal volume of 0.5% sodium dodecylsulphate, 150 mM sodium acetate pH 6.0 was added and the ^{32}P-labelled poly(dT) was precipitated with two volumes of ethanol.

Abbreviations. Poly(U) polyuridylic acid, poly(A) polyadenylic acid, poly(dT) polythymidylic acid; mRNA, messenger RNA.

Enzymes. Alkaline phosphatase (EC 3.1.3.1); polynucleotide kinase (EC 2.7.1.–).

Definition. A_{260} unit, the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 260 nm, when measured in a 1-cm path-length cell.

Sucrose Gradients

7—25% sucrose gradients were prepared in 50 mM Tris-HCl pH 7.6 at 20 °C, 150 mM NaCl, 1 mM EDTA and 0.5% sodium dodecylsulphate. 10 μg reticulocyte RNA was dissolved in 100 μl gradient buffer minus sucrose and mixed with 0.01—1.0 μg poly(U) and layered onto a 5-ml gradient. Gradients were centrifuged in the SW 50.1 rotor of the Spinco Model L centrifuge for 2.5 h at 45000 rev./min (189000 × g_{av}) at 20 °C. Gradients were continuously monitored for ultraviolet absorbance at 254 nm with an Isco 222 Analyser. Fractions of 0.13 ml were mixed with 0.3 ml of 0.5 M NH_4OH, 0.5 mM EDTA and dried at 60 °C in scintillation vials. 0.3 ml water and 7 ml scintillation liquid [0.5% (w/v) butyl-PDB, 40% (v/v) methoxyethanol, 60% (v/v) toluene] were added and ^3H radioactivity was determined in the Packard Tri-Carb scintillation spectrometer.

Polyacrylamide-Gel Electrophoresis

Reticulocyte RNA and poly(dT) were dissolved in double-strength electrophoresis buffer (electrophoresis buffer in 36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA, 0.2% sodium dodecylsulphate) containing 14% sucrose. 10 to 50 μg reticulocyte RNA were mixed with varying amounts of poly(U) or poly(dT) and the mixture was fractionated on a 2.4% or 3.0% gel at 5 mA/gel, 7 V/cm gel-length for 3 h [10,11]. Gels were scanned for ultraviolet absorbance at 265 nm in a Joyce-Loebl UV Gel Scanner, frozen in solid CO_2 and sliced transversely at 0.8-mm intervals with a Mickle Gel Slicer. Slices of gels containing ^{32}P were dried and counted in a Geiger tube apparatus with a background of 6 counts per minute [8]. Slices of gels with [^3H]poly(U) were incubated with NH_4OH, mixed with scintillator and counted as above.

RESULTS

Fractionation by Sedimentation Analysis

The sedimentation coefficient of [^3H]poly(U) in the absence of any other added RNA was 3 S to 6 S, as shown in Fig. 1 A. When mixed with the reticulocyte RNA, some poly(U) sedimented with very much higher sedimentation coefficients. This is taken to indicate binding of the poly(U) to poly(A) sequences in RNA to form faster-sedimenting hybrids. The distribution of poly(U) on the gradient depended on the ratio of poly(U) to reticulocyte RNA applied (Fig. 1).

When 1 μg poly(U) was mixed with 10 μg reticulocyte RNA, only a small proportion of the poly(U) was bound to the RNA and most remained unbound at the top of the gradient (Fig. 1 A). A peak of radioactivity at about 10 S, which was assumed to be hybridisation of the poly(U) with the 10-S mRNA for haemoglobin, was visible but was partly obscured by the tail of poly(U) counts stretching from the top of the gradient.

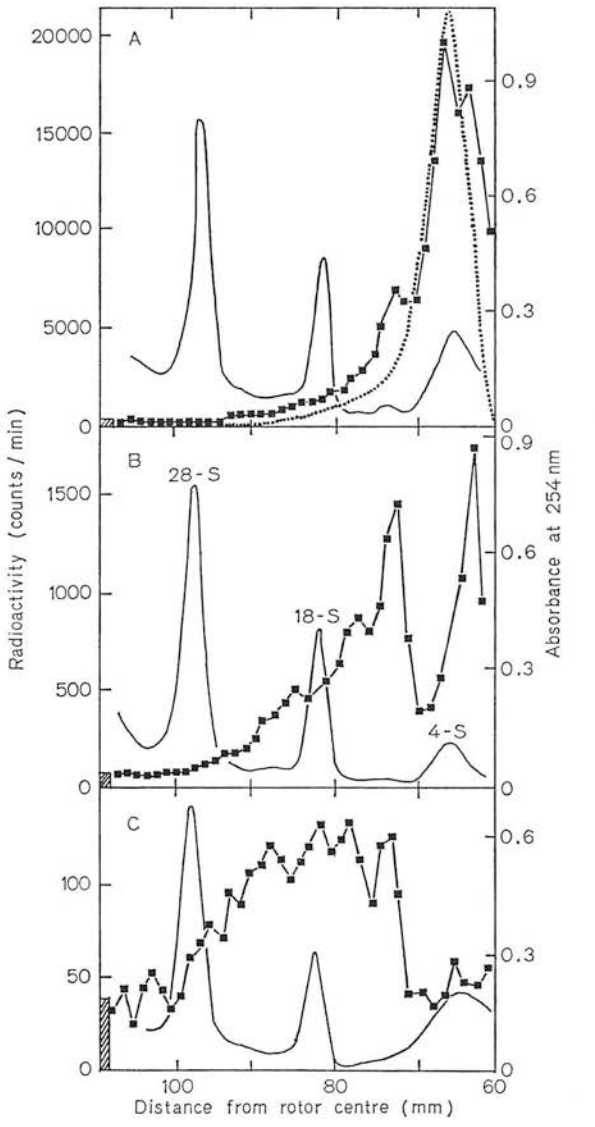

Fig. 1. *Sucrose density-gradient profiles of mixtures of 10 μg reticulocyte RNA and [^3H]poly(U).* (A) 1 μg [^3H]poly(U), (B) 0.1 μg [^3H]poly(U) and (C) 0.01 μg [^3H]poly(U). The continuous line is absorbance at 254 nm (■——■) radioactivity; the dotted line in (A) is the radioactivity profile from sedimentation of 1.0 μg [^3H]poly(U) in the absence of reticulocyte RNA

When 0.1 μg poly(U) was mixed with 10 μg reticulocyte RNA, about 30% of the poly(U) remained as free poly(U) near the top of the gradient (Fig. 1 B). The peak of radioactivity at 10 S was now prominent and smaller peaks at about 13 S and 20 S were also apparent. (These S values were estimated using the 18-S and 4-S RNAs as markers.) There was no

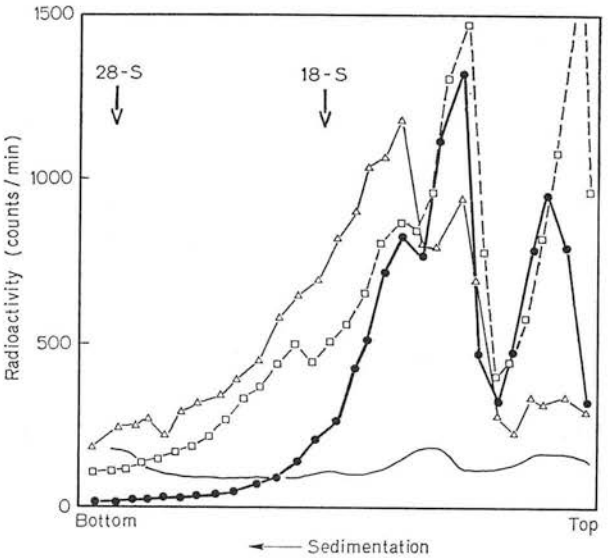

Fig. 2. *Sucrose density-gradient sedimentation of mixtures of reticulocyte RNA and [³H]poly(U).* (□— —□) Radioactivity profile of 10 μg total reticulocyte RNA and 0.1 μg [³H]poly-(U); (●——●) Radioactivity profile of 10-S to 12-S fraction prepared from 10 μg reticulocyte RNA and 0.08 μg [³H]poly-(U); (△——△) radioactivity profile of 10-S to 12-S fraction prepared from 10 μg reticulocyte RNA and 8 ng [³H]poly(U), plotted with the radioactivity multiplied ten times. Continuous line: absorbance at 254 nm of 10-S to 12-S fraction of reticulocyte RNA

binding of poly(U) with 18-S or 28-S ribosomal RNA but there was clearly some binding with a polydisperse distribution.

With a very low ratio of poly(U) to reticulocyte RNA, using only 0.01 μg poly(U) to 10 μg reticulocyte RNA (Fig. 1C) almost all of the poly(U) sedimented faster than free poly(U). The peak of binding at 10 S was identifiable but it is clear that under these conditions of limiting poly(U) concentration, most poly(U) sedimented faster than the 10-S haemoglobin mRNA.

Fig. 2 shows that this effect was probably a result of network formation, that is by two or more molecules of RNA which contain poly(A) sequences hybridising to a single poly(U) molecule. A 10-S to 12-S fraction of reticulocyte RNA was prepared by alcohol precipitation of the appropriate fractions of total RNA sedimented through a sucrose gradient. This RNA fraction was dissolved in gradient buffer, mixed with varying amounts of poly(U) and resedimented through sucrose gradients.

Under saturating conditions of poly(U), *i.e.* where appreciable free poly(U) remained at the top of the gradient, the vast majority of the hybridised, faster-sedimenting poly(U) was associated with the 10-S and 12-S mRNA's and coincided with the peak of ultraviolet absorbance on the gradient. Very little poly(U) sedimented faster than 18 S. When one tenth as much poly(U) was mixed with the same amount of 10-S to 12-S RNA, little free poly(U) remained at the top of the gradient after sedimentation. Relatively few counts were associated with the 10-S peak. Most of the counts were to be found in the 12-S to 15-S region and a considerable proportion of the radioactivity was found in regions heavier than 18-S.

We conclude that under conditions of limiting poly(U) concentration, association of one poly(U) molecule with more than one RNA molecule may result in a spurious distribution of counts in the heavier regions of the gradient, but that under saturating poly(U) concentrations, network formation does not occur to a significant extent. The poly(U) found sedimenting faster than 18-S, when total RNA was mixed with saturating amounts of poly(U), therefore showed genuine "monogamous" hybridisation of poly(U) to fast-sedimenting RNA. A parallel may be drawn here between the poly(U) · RNA complexes sedimenting at 13-S and 20-S (Fig. 1B) and the presumptive mRNA components reported by others at 12-S [10, 12, 13] and 17-S [14].

Fractionation by Gel Electrophoresis

We attempted to fractionate similar reticulocyte RNA plus poly(U) mixtures by polyacrylamide-gel electrophoresis. Fig. 3A shows that poly(U) was bound in a peak with an electrophoretic mobility similar to that expected for the haemoglobin mRNA. This corresponded to the peak of poly(U) binding at the 10-S position found on gradients. Fig. 3A also shows that there were two peaks of binding of poly(U) to heavier RNA's, with electrophoretic mobilities slightly greater than the 28-S and 18-S ribosomal RNA's, respectively. These correspond to the two peaks of poly(U) binding in the heavier region of gradients (Fig. 1B). There was a very high level of background or polydispersed poly(U) labelling on the gel.

From its sedimentation coefficient and molecular weight, the poly(U) used for these experiments would be expected to have an electrophoretic mobility close to that of transfer RNA [11]. Electrophoresis of poly(U) alone (Fig. 3B) showed that few counts moved as fast as transfer RNA; most of the poly(U) had a much lower mobility and gave a polydisperse distribution on the gel similar to that obtained when mixed with reticulocyte RNA. We found that this distribution was very little altered by partial hydrolysis of the poly(U) by alkali. We therefore conclude that the mobility of poly(U) alone is more or less independent of its molecular weight and that it binds to polyacrylamide gels under these conditions.

Poly(dT) did not bind to the gel in this manner, but migrated as a single, fairly sharp peak with a mobility slightly lower than that of 5-S RNA (Fig. 4A).

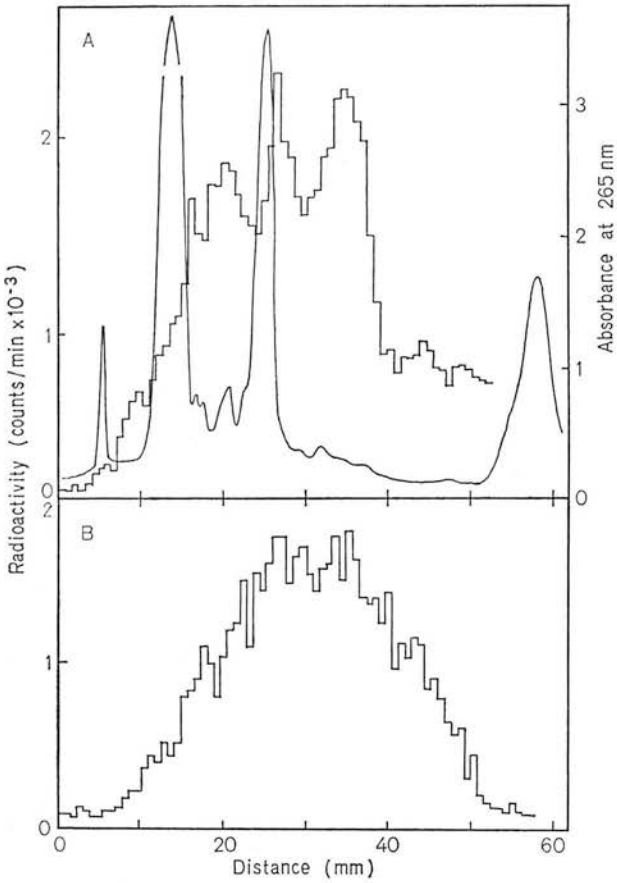

Fig. 3. *Electrophoresis, on 2.4% polyacrylamide gels, of (A) about 25 µg reticulocyte RNA together with 0.4 µg [³H]poly(U) (B) 0.4 µg [³H]poly(U) alone.* The continuous line shows ultraviolet absorbance: the peaks are 28-S ribosomal RNA at 14 mm; 18-S ribosomal RNA at 25 mm and transfer RNA at 58 mm. The histogram shows ³H radioactivity: the peak at 34 mm is interpreted as [³H]poly(U) bound to the 10-S haemoglobin mRNA

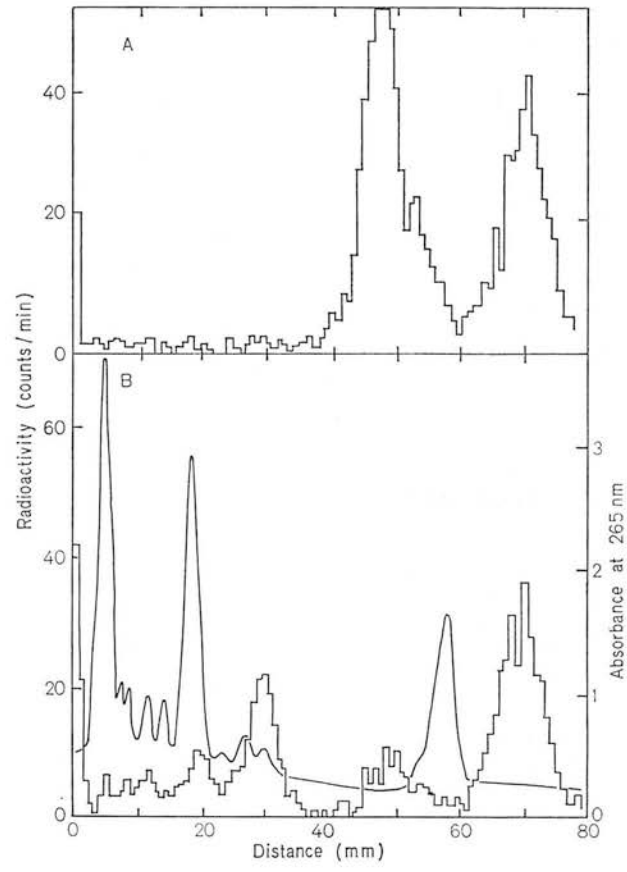

Fig. 4. *Electrophoresis, on 3% polyacrylamide gels, of (A) [³²P]poly(dT) alone, (B) about 25 µg reticulocyte RNA together with [³²P]poly(dT).* The continuous curve shows ultraviolet absorbance: the peaks are 28-S ribosomal RNA at 5 mm, 18-S ribosomal RNA at 19 mm and transfer RNA at 59 mm. The histogram shows ³²P radioactivity: free [³²P]-poly(dT) at 50 mm and poly(dT) bound to 10-S haemoglobin mRNA at 29 mm

The electrophoretic mobility of the poly(dT) was in fact exceptionally low in view of its sedimentation coefficient of 2-S (Miles). The residual [³²P]ATP from the labelling of poly(dT) migrated slightly faster than the transfer RNA.

Electrophoresis of poly(dT) mixed with reticulocyte RNA resulted in three radioactivity peaks migrating slower than the poly(dT) (Fig. 4B). This result therefore parallels the three fast-sedimenting radioactive peaks found in poly(U):reticulocyte-RNA mixtures (Fig. 1A). We interpret the most prominent of the poly(dT) labelled peaks, at 30 mm into the gel in Fig. 4B, as binding of the poly(dT) to the 10-S haemoglobin mRNA. The absorbance profile of reticulocyte RNA run alone shows the 10-S haemoglobin mRNA (Fig. 5) at 33 mm into the gel. The presence of poly(dT) during electrophoresis resulted in a shift of the 10-S absorbance peak from 33 mm to a region of slightly lower electrophoretic mobility, at 29 mm (Fig. 5). This was coincident with the poly(dT) radioactivity peak. Such a decrease in electrophoretic mobility would be expected as a result of the increased molecular weight of the mRNA after addition of the poly(dT) molecule. The apparent molecular weight of the mRNA · poly(dT) complex was about 300000, calculated from its electrophoretic mobility [11] and using the 5-S and 18-S ribosomal RNA as standards. This value is slightly higher than the sum of the molecular weights, measured by electrophoretic mobility, of the poly(dT) (60000, Fig. 4) and the 10-S mRNA in the absence of poly(dT) (210000, Fig. 5).

DISCUSSION

This method allows a very simple characterisation of the distribution of those RNA molecules

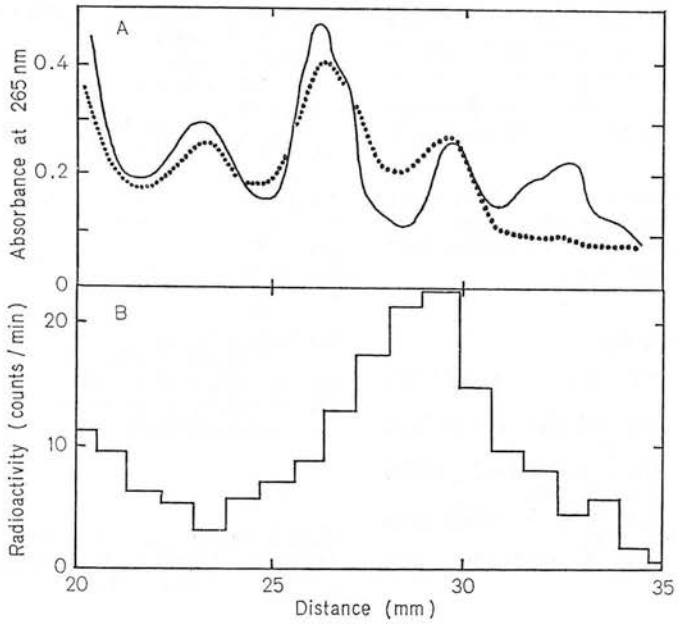

Fig. 5. *Electrophoresis of reticulocyte RNA with and without [^{32}P]poly(dT): expansion of the region from 20—35 mm in Fig. 4B.* (A) Ultraviolet absorbance scans of (——) reticulocyte RNA alone: the peak at 33 mm is the 10-S haemoglobin mRNA and (·····) reticulocyte RNA run together with [^{32}P]poly(dT). (B) ^{32}P radioactivity on gel of reticulocyte RNA plus poly(dT). The peak at 29 mm is the hybrid of poly(dT) and 10-S mRNA

which contain poly(A) sequences. It is not necessary to label the cells or the RNA. The method detects such RNA molecules independently of their rate of turnover in the cell. The usefulness of the technique will obviously depend on the extent to which poly(A) sequences occur in mRNA and in nuclear heterogeneous or other RNA's. So far, poly(A) sequences have been found in mammalian RNA, in *Xenopus* RNA (Rosbash and Ford, personal communication), in yeast RNA (Creanor and Fraser, unpublished results) and it has long been known that heterogeneous RNA from plant tissues has a very high adenosine content [15]. On the other hand, it is known that poly(A) sequences are not present in two sorts of identifiable mRNA molecules: those for histones [16] and tobacco mosaic virus RNA (Fraser, unpublished results).

The method promises to be a means of estimating the total amount of messenger RNA in a cell or tissue, by titrating the adenosine-rich sequences with radioactive polynucleotides, as is illustrated in Fig. 1. We found that a maximum of 0.025 μg poly(U) was bound to the haemoglobin mRNA which is contained in 10 μg total reticulocyte RNA. If we assume that this represents 0.1 μg haemoglobin mRNA [12] which has a molecular weight of about 200000 (Fig. 5) [12, 17] and if the molecular weight of the poly(U) is 50000, then one poly(U) molecule has been bound per mRNA molecule. This result suggests that the amount of poly(U) binding may be used as a rough assay of the amount of messenger RNA present, including mRNA molecules which have a poly-disperse distribution on gradients or gels.

For both the assay of the amount of messenger present and characterisation of size distribution of mRNAs, it is important that the homopolymer must be present in small excess. This avoids spurious results from network formation which occur under limiting homopolynucleotide concentrations and unclear results which occur under conditions of vast polynucleotide excess. To avoid network formation, it would undoubtedly be of advantage to use a radioactive homopolynucleotide with the shortest chain length consistent with formation of stable hybrids with poly(A) regions.

An alternative approach for the characterisation of RNA which contains poly(A) sequences is to fractionate the RNA on sucrose gradients or by gel electrophoresis and then to challenge each fraction with labelled poly(U) (J. Bishop, personal communication). This procedure does not alter the fractionation properties of the RNA. It is possible to use ribonuclease to digest away any free poly(U) molecules and to obtain exact measures of the amount hybridised. The technique we describe here is, however, much simpler, and under suitable conditions neither background counts nor network formation interfere with the results. Furthermore, the increase in apparent molecular weight of the mRNA in the presence of poly(U) or poly(dT) can help to identify

the true absorbance peaks of messenger RNA and distinguish them from the very many other minor RNA components. Such a shift of absorbance peaks can, of course, only be seen by gel electrophoresis and not on sucrose gradients which lack the necessary resolving power.

This work was supported by the Medical Research Council. We thank Miss Anne Baker and Mr Harry Urquhart for capable technical assistance and Mr Jonathan Slack for assistance with labelling of poly(dT).

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Journal of Experimental Botany, Vol. 32, No. 131, pp. 1223–1230, December 1981

A Rapid and Simple Radioassay for Abscisic Acid using ^{14}C-Diazomethane

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Received 12 June 1981

ABSTRACT

A simple physical method for measuring abscisic acid concentration in plant material is described. Abscisic acid in partially purified extracts was radiolabelled by reaction with ^{14}C-diazomethane to give ^{14}C-methyl abscisate, which was purified from other radiolabelled products by thin layer chromatography. Abscisic acid concentration was measured by comparison of the ^{14}C radioactivity incorporated into plant abscisic acid with that in standard ^{14}C-methyl abscisate prepared under the same conditions from known amounts of pure abscisic acid. Losses of abscisic acid which occurred during purification were corrected by measuring the recovery of ^{3}H-abscisic acid added to initial extracts.

Abscisic acid concentration was measured by radioassay and by conventional electron capture–gas chromatography in oat, bean, and turgid or wilted tobacco leaves. Results from the two methods were closely comparable. Radioassay is as rapid and sensitive as existing procedures for measuring abscisic acid, but requires only simple and inexpensive chromatographic equipment.

INTRODUCTION

Abscisic acid (ABA) is a naturally occurring hormone important in control of growth, senescence and water status in plants (reviewed by Letham, Goodwin, and Higgins, 1978; Walton, 1980). It can be assayed by gas chromatography (GC), high pressure liquid chromatography (HPLC), and radioimmunoassay (RIA) (Seeley and Powell, 1970; Ciha, Brenner, and Brun, 1977; Walton, Dashek, and Galson, 1979; Weiler, 1979).

In this paper we describe a novel physical method for measuring ABA concentration in plant material. The method is rapid, simple, and highly sensitive; it does not involve immunological procedures or complex chromatographic equipment.

MATERIALS AND METHODS

Plants

Tobacco (*Nicotiana tabacum* L. cv. White Burley), bean (*Phaseolus vulgaris* L. cv. The Prince), and oat (*Avena sativa* L. cv. Osprey) plants were grown in Levington Universal compost under normal glasshouse conditions. Leaves were detached from tobacco plants and wilted for various times in a stream of warm air to produce a wide range of endogenous ABA concentrations; percentage loss in fresh weight in the different treatments was recorded. All harvests were made when plants were 6–10 weeks old.

ABA extraction and initial purification

Leaf samples of 1–2 g fresh weight were homogenized at 0 °C in 10 ml 80% (v/v) methanol containing 10 mg l^{-1} butylated hydroxytoluene. Where it was required to measure the percentage recovery of ABA after purification, ^{3}H-ABA (DL-*cis, trans*[G-^{3}H]abscisic acid: 888 GBq $mmol^{-1}$; Radiochemical Centre, Amersham; diluted with unlabelled *cis, trans*-ABA to a specific activity of 418 MBq $mmol^{-1}$) was added to samples prior to homogenization.

The homogenate was centrifuged at 2000 *g* for 5 min, and methanol removed from the supernatant *in vacuo*. The aqueous residue was successively partitioned with dichloromethane at pH 8·3 and 3·0 (Whenham and Fraser, 1981). The acid dichloromethane fraction containing ABA was dried over anhydrous sodium sulphate and redissolved in methanol. Extracts were purified by thin layer chromatography (TLC) on silica gel plates (Whatman LK6DF with preabsorbent zone) developed twice with toluene/ethyl acetate/glacial acetic acid (25:15:2, by vol.) with (±)-*cis, trans*-ABA (Sigma Chemical Co.) as marker. Zones on chromatograms corresponding to *cis, trans*-ABA were located under ultraviolet light and eluted with methanol.

Radioassay

1·85 MBq (1·03 mg) of ^{14}C-diazald (*N*-[^{14}C]methyl-*N*-nitroso-*p*-toluene-sulphonamide; 385 MBq $mmol^{-1}$; Radiochemical Centre, Amersham) was added to unlabelled diazald (25 mg) in 5 ml ether/carbitol (3:2, by vol.). The ^{14}C-diazald was stable indefinitely if stored dry at −20 °C; solutions were stored at −20 °C for several weeks without detectable degradation.

^{14}C-Diazomethane was liberated from the ^{14}C-diazald solution by addition of 1 ml 60% (w/v) potassium hydroxide, and dissolved in 5 ml ether/methanol (9:1, by vol) (Schlenk and Gellerman, 1960). Ethereal diazomethane was added to ABA extracted from leaves and to known weights of pure *cis, trans*-ABA in sealed vials, in sufficient excess to give quantitative esterification of the ABA present.

Radiolabelled methyl abscisate (MeABA) was separated from other radiolabelled reaction products by TLC on silica developed twice with hexane/ethyl acetate (3:1, by vol.) containing 200 mg l^{-1} butylated hydroxytoluene. Zones on chromatograms containing MeABA were located under ultraviolet light and eluted with ethyl acetate. A sample of the eluate was mixed with 5 ml scintillation liquid (5 g 2-(4′-tert-butylphenyl-5-(4″-biphenylyl)-1,3,4-oxadiazole l^{-1} toluene) and counted in an LKB Rackbeta liquid scintillation counter. Correction of counts to 100% counting efficiency was by the external standard channels ratio method. Where ^{3}H-ABA was used as recovery standard, ^{14}C and ^{3}H radioactivity were measured in the same sample by dual isotope counting. Counts in each channel were corrected for cross-over between channels, and to 100% counting efficiency for each isotope, by the automatic dual channel quench correction programme.

ABA concentrations in leaf samples were calculated using the equation:

$$\text{ABA (ng g}^{-1}) = \frac{\left(\frac{A \times B}{C \times D}\right) - E}{F}$$

where A = ^{14}C radioactivity (d min^{-1}) in the leaf sample

B = ^{3}H radioactivity (d min^{-1}) in the standard ABA added at the beginning of extraction

C = ^{14}C radioactivity (d min^{-1}) per ng pure *cis, trans*-ABA radiolabelled under the same conditions

D = ^{3}H radioactivity (d min^{-1}) recovered in the purified MeABA

E = ng ^{3}H-ABA added at the beginning of extraction

F = fresh weight of leaf sample.

In words, the equation uses the ^{3}H-ABA recovery values to correct ^{14}C-ABA radioactivity to 100% recovery, and converts ABA ^{14}C radioactivity to ABA weight using the ^{14}C radioactivity of a known amount of pure *cis, trans*-ABA radiolabelled under the same conditions. The weight of leaf ABA is found by subtracting the weight of the ^{3}H-ABA recovery standard added at the start of purification, and concentration is calculated by dividing by sample fresh weight.

Electron capture–gas chromatography (EC–GC)

ABA concentration in samples prepared for radioassay was also measured by gas chromatography, using a 2 m column of 2·5% OV-1/Gas-Chrom Q at 235 °C. Injector and detector were maintained at 300 °C. The carrier gas was argon/methane (95:5) at a flow rate of 30

ml min^{-1} through the column (Whenham and Fraser, 1981). The electron capture detector was calibrated by chromatography of known amounts of MeABA (pure *cis, trans*-ABA, methylated with unlabelled diazomethane).

The identification of ABA on chromatograms was confirmed by mass spectrometry. Individual samples prepared as for EC–GC were combined to obtain sufficient material. A Kratos model 902 spectrometer was used, with a scan rate of 9 s per decade, source temperature 200 °C, and ionization energy 70 eV. Samples were analysed by direct probe, or by combined gas chromatography–mass spectrometry (GC–MS), using a column of 3% OV-1 and flame-ionization detector.

The combined GC–MS of *cis, trans*-MeABA purified from leaves agreed closely with the published mass spectrum of pure MeABA (Gray, Mallaby, Ryback, and Williams, 1974). The specific activity of the ^{14}C-diazomethane used in the methylation reaction was not high enough to cause detectable alteration in the mass spectrum. The total ion current from *m/e* 51 to *m/e* 281, measured in the middle of the *cis, trans*-MeABA peak was 55 567. The ion current due to those ions also present in the mass spectrum of pure standard *cis, trans*-MeABA was 43 124, indicating an MS purity of the peak of 78%. However, this is a serious underestimate of the purity, as no allowance could be made for the background contribution to the total ion current due to column bleed. The most abundant contaminating ion in the peak not due to MeABA was mass 131; its ion current was only 1% of the combined ion currents of the MeABA-derived ions. These data therefore indicate that electron capture–gas chromatography was measuring authentic ABA in the absence of significant levels of contaminants.

RESULTS AND DISCUSSION

Radioassay of ABA

Figure 1 shows that the ^{14}C-methylation reaction was linear over a very wide range of ABA concentration. The equilibrium of the reaction strongly favours formation of the methyl ester. Control experiments showed that in the presence of excess diazomethane, esterification was quantitative (more than 95%). In practice, the amount of ^{14}C-diazomethane added to each sample was an excess of that required for complete esterification of the known or expected ABA content.

Success of the radioassay depended with leaf samples on completely purifying the ^{14}C-MeABA from all other components in the extract which could become radiolabelled. The initial purification of free ABA before radiolabelling, by solvent partition and TLC, removed many interfering substances. These included compounds closely related to ABA such as glucosyl-ABA, *trans, trans*-ABA, MeABA, phaseic acid, and dihydrophaseic acid (Zeevaart and Milborrow, 1976; Weiler, 1980).

The choice of TLC conditions used to purify ^{14}C-MeABA in the second TLC stage was critical. TLC on silica, multiply developed with hexane/ethyl acetate, separated ^{14}C-MeABA completely from other radiolabelled reaction products. The purity of the ^{14}C-MeABA prepared by TLC is clearly central to the reliability of the method, and was therefore established in several ways.

(1) The product gave only a single radioactive spot, with a mobility corresponding to that of authentic MeABA, when eluted and refractionated by TLC using five different solvent systems: hexane/ethyl acetate (3:1); dichloromethane/methanol (85:15); hexane/acetone (1:1); n-butanol/16 N ammonium hydroxide/water (86:5:14), and toluene/ethyl acetate/acetic acid (50:30:4). Any significant amounts of contaminating radiolabelled products would have been detected as separate radiolabelled spots on at least one of these solvent systems.

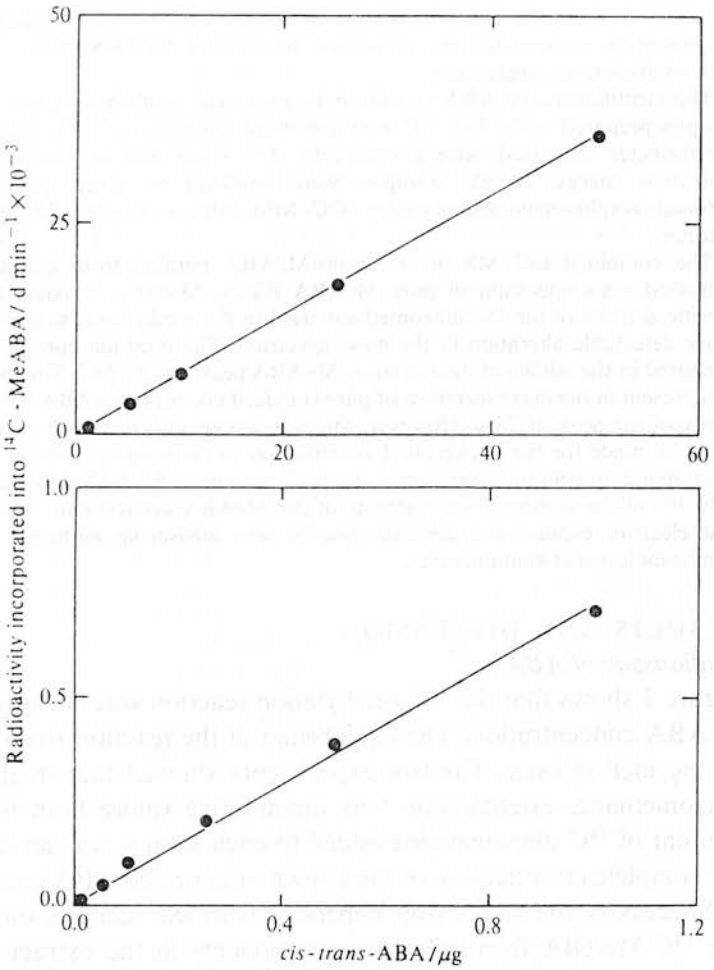

FIG. 1. Standard curve for abscisic acid radioassay. Pure *cis*, *trans*-^{3}H-ABA (5 ng–50 μg, specific activity 100 MBq mmol^{-1}) was ^{14}C-labelled with ^{14}C-diazomethane and purified by TLC in hexane/ethyl acetate. ^{14}C-radioactivity incorporated into MeABA was corrected to 100% recovery using the ^{3}H recovery measurements.

(2) Radiolabelled MeABA was eluted from the TLC plate and successively rechromatographed using each of the five solvent systems in turn. The specific activity of the starting material (radioactivity per unit weight of MeABA, measured by EC–GC) was 4·1 MBq mmol^{-1}; after the five further chromatogaphic steps it was 4·4 MBq mmol^{-1}, i.e. not significantly different. Any contaminating radiolabelled products in the starting material would have been removed by the five further chromatographic steps, and this would have been detected as a reduction in specific activity of the ^{14}C-MeABA after these steps.

(3) Fractionation of the ^{14}C-MeABA by gas chromatography showed a major peak of *cis*, *trans*-MeABA, and a minor peak of *trans*, *trans*-MeABA. The *trans*, *trans*-MeABA was normally less than 5% of the *cis*, *trans*-MeABA, and arose by light-stimulated isomerization of ABA during the second TLC

stage. The flame-ionization detector in particular would have revealed any significant amounts of other C–H containing compounds, but minor peaks on the chromatograms accounted for less than 5% of the MeABA peaks. Combined GC–MS showed that the ^{14}C-MeABA was not contaminated by significant amounts of other compounds which co-chromatographed with the *cis*, *trans*-MeABA peak.

(4) With direct probe assay mass spectrometry, the whole sample is assayed without prior fractionation by GC. The mass spectrum of ^{14}C-MeABA by direct probe assay was again closely comparable with the published mass spectrum of pure MeABA (Gray *et al.*, 1974). Purity was estimated to be close to 100%, but could not be more precisely determined because of difficulties in quantifying background. The mass spectrum showed no sign of major contamination by ions not derived from MeABA.

(5) The specific activity of ^{14}C-MeABA prepared from leaf extracts was the same as that of ^{14}C-MeABA prepared from pure *cis,trans*-ABA radiomethylated under the same conditions. If the leaf extract had contained significant amounts of radiolabelled compounds other than ^{14}C-MeABA, this would have shown as an apparent specific activity higher than that of pure MeABA.

We consider that these results provide conclusive proof that the purification procedure gave complete separation of ^{14}C-MeABA from other radiolabelled reaction products. Several other TLC/solvent systems were tried, but were found to be less effective.

Radioassay was used to measure ABA concentration in turgid and wilted tobacco leaves, and the results compared with conventional EC–GC. After radiolabelling with ^{14}C-diazomethane and purification by TLC, samples were divided. The ABA content of one portion was measured by radioactivity, and of the other by GC. Losses of ABA which occurred during extraction and purification thus affected both methods equally in this experiment: no radioactive recovery standard was added.

Both radioassay and EC–GC gave very similar values for ABA concentration of individual samples, over a very wide range (50-fold) of endogenous concentration (Table 1). Estimates of ABA concentration from the two assays differed by 7 $\pm$ 1% over the whole concentration range. These results therefore show that radioassay is a reliable method for estimating ABA concentration.

Use of ^{3}H-ABA as recovery standard

A problem affecting both radioassay and EC–GC is loss of ABA which occurs during purification. Table 1 shows that, with either method, replicate samples from leaves which had received the same treatment showed considerable variation in measured ABA concentration: much of this variation can be ascribed to variation in ABA recovery. For accurate analysis, therefore, a recovery standard must be added to each extract to allow correction of losses. ^{14}C-ABA is a convenient standard for EC–GC (Whenham and Fraser, 1981) but obviously cannot be used in radioassays employing ^{14}C-diazomethane. Instead, ^{3}H-ABA (Walton, Wellner,

TABLE 1. *ABA concentration in extracts from turgid and wilted tobacco leaves, measured by radioassay and electron capture–gas chromatography*

Loss in fresh weight (%)	ABA/ng g^{-1} fr. wt.	
	radioassay	electron capture
0	10	10
	18	16
10	46	48
	62	66
25	386	406
	485	533
	402	409
	481	540
	256	214
	367	391
	249	257
	420	466

and Horgan, 1977) was used as recovery standard for radioassay. Control experiments showed that the specific activity of ^{3}H-ABA was unaffected by the purification methods used: loss of radioactivity by ^{3}H exchange therefore did not occur.

Use of ^{3}H-ABA as recovery standard for the ^{14}C radioassay required dual isotope counting of the final sample. In practice, it proved simple to establish counting parameters which allowed reliable correction for cross-over between the ^{3}H and ^{14}C channels, and for quenching in each channel. Control experiments showed that the additional errors in measurement of either ^{3}H or ^{14}C radioactivity introduced by simultaneous measurement of the other isotope were negligible.

^{3}H-ABA added to extracts also acted as a carrier, and increased the percentage recovery of ABA during purification, particularly from tissues with low endogenous ABA concentrations (Milborrow, 1978). However, for reliable results, it was important that the amount of ^{3}H-ABA added did not exceed the endogenous ABA content of the sample.

Table 2 shows a comparison of measurements of ABA concentration in three

TABLE 2. *Measurement of endogenous ABA concentrations in leaves of three species by radioassay and electron capture–gas chromatography*

^{3}H-ABA was used as recovery standard for all assays. Values are means ± standard errors.

Species	ABA concentration/ng g^{-1} fr. wt.		Number of determinations by each method
	by radioassay	by EC–GC	
Oat	44 ± 6	36 ± 6	4
Bean	37 ± 7	31 ± 10	3
Tobacco (wilted)	264 ± 11	235 ± 7	8

species, by radioassay and EC–GC. In all cases, ^{3}H-ABA was used as recovery standard. It is clear that both methods gave very similar answers for each species. Furthermore, the magnitude of the errors was similar for both methods. These results therefore suggest that radioassay, using ^{3}H-ABA as recovery standard, is a reliable method for estimating ABA concentration in leaves.

Comparison of radioassay with other methods

Under the ^{14}C-labelling conditions used, the radioassay detection limit for ABA in purified leaf samples was 4 ng, giving a count rate of twice background. This compares well with the sensitivity of EC–GC and HPLC, both of which require about 2 ng ABA per purified sample (Ciha *et al.*, 1977). Recently, a radioimmunoassay has been described with a lower measurement limit of 53 pg ABA (Weiler, 1980); this appears to be the most sensitive method currently available. The sensitivity of the radioassay described here can be increased to 150 pg ABA per sample, by using the ^{14}C-diazald undiluted with carrier. With this degree of sensitivity, radioassay is capable of measuring the lowest ABA concentrations yet reported to occur naturally (Milborrow, 1978) while still using conveniently small samples of 1 g or less. For the great majority of experimental applications, however, use of ^{14}C-diazald diluted with non-radioactive carrier gives sufficient sensitivity.

Radioassay has some useful advantages over other techniques for measuring ABA concentration. It is at least as rapid and sensitive as EC–GC and HPLC, but requires only simple and inexpensive chromatographic equipment. Radioassay does require a scintillation counter, but so do RIA, and EC–GC or HPLC when a radioactive recovery standard is used.

EC–GC requires frequent detector standardization to ensure reliable results, particularly when only partially purified extracts are analysed (Quarrie, 1978). Radioassay needs only a single standardization for each batch of ^{14}C-diazald. Obviously, before use of radioassay with any previously untried species, it would be necessary to check that the second TLC stage did separate ^{14}C-MeABA from other radiolabelled compounds.

Until recently, sera against ABA lacked specificity: ABA esters, phaseic acid, and dihydrophaseic acid had to be removed by solvent partition and chromatographic steps before assay of ABA by RIA (Walton *et al.*, 1977). Highly specific antisera for ABA have now been produced (Weiler, 1980) which can be used with crude extracts of leaf. With this, RIA is more rapid than radioassay for individual measurements, but production of the antisera requires complex immunological procedures. If groups of samples are produced together by radioassay, the mean process time per sample is about 30 min, and all reagents are immediately available. Cost of reagents per sample is minimal. RIA also has a rather limited linear response range (Walton *et al.*, 1979; Weiler, 1979, 1980): in contrast radioassay gives a linear response over a very wide range of ABA concentration (Fig. 1).

With suitable modification of the extraction and TLC stages, it is probable that radioassay could be used to measure, with the same sensitivity, the concentration of

other compounds related to ABA, such as phaseic acid and dihydrophaseic acid. 'Bound' ABA (ABA glucosyl ester; Milborrow, 1970) can also be measured by radioassay after conversion to free ABA by alkaline hydrolysis.

ACKNOWLEDGEMENT

We thank John Eagles of the Food Research Institute, Norwich, for mass spectrometric authentication of ABA.

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